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(57) Abstract

Sialyls Lewis (sLe) antigens are functionally important, immunogenic, tumorigenic or differentiation antigens and potential targets for both passive and active specific immunotherapy of melanoma and other cancers sharing these antigens. The present invention concerns the use of such antigens in vaccine formulations for the treatment of a variety of cancers and in particular melanoma. The B lymphocytes from the vaccine recipients will be used to harvest human monoclonal antibodies and use it as a drug for treatment of melanoma and other cancers.

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DESCRIPTION

SIALYL LEWIS ANTIGENS AS TARGETS FOR IMMUNOTHERAPY

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates generally to the field of cancer vaccine immunotherapy. More particularly, it concerns the use of Sialyl Lewis antigens in vaccine formulations for the treatment of a variety of cancers and in particular, melanoma.

2. Description of Related Art

The concept of using vaccines to induce specific immunity against cancer has existed since the turn of the century, when cancer therapists were first attracted by the success of vaccines in inducing active immunity against infectious diseases. Cancer vaccines differ from vaccines against infectious diseases in that they are administered as therapy after the advent of disease, rather than prophylactically before the disease develops. The theory behind vaccines for cancer and infectious diseases is, however, similar. Both seek to stimulate the patient's own immune system to fight the disease through the introduction of killed whole organisms or cells, specific subcellular or purified antigens, non-pathologic strains of living organisms or their derivatives or tumor cells.

Since cancer vaccines stimulate antitumor immune responses and effectively increase resistance to cancer in preclinical animal models, active immunotherapy with cancer vaccines has been accepted as an experimental treatment modality of certain cancers. The growth of tumor not only depends on the intrinsic properties of the tumor but also is influenced by host defense properties. The foundation for active specific immunotherapy is based on evidence documenting interactions between tumor and immune system. The supporting observations include complete spontaneous regression of widely disseminated cancers, partial regression of established disease, prolonged *in situ* latent period prior to metastasis, prolonged interval between tumor excision and recurrence, circulating tumor cells without metastatic spread, and metastatic cancer without known primary (Morton and Barth, 1996).

Of more than fifty trials conducted in cancer patients, the majority involved melanoma patients, receiving whole cell autologous (Morton and Ravindranath, 1996) or allogeneic

(Morton *et al.*, 1984) vaccines, viral lysates of melanoma cells (Wallack *et al.*, 1995), antigens shed from cultured melanoma cells (Bystryn *et al.*, 1995) or purified melanoma-associated antigens (Livingston, 1995). Among the clinical parameters monitored after vaccine treatment were complete response with no evidence of disease, partial response, disease free-survival, overall survival, delayed type of hypersensitivity reaction, and antibody (IgM / IgG) responses (Bystryn *et al.*, 1995; Morton and Ravindranath, 1996; Morton *et al.*, 1992, 1994; Wallack *et al.*, 1995).

Studies at the John Wayne Cancer Institute with polyvalent Melanoma Cell Vaccine (pMCV) have demonstrated in a large Phase II trial that the median survival and five-year survival of patients receiving this vaccine have significantly increased compared to historical control patients after correction for known prognostic factors. A Phase III randomized multi-center trial is currently underway to confirm this observation. However, even if this multicenter trial is successful, there is still a large rate of treatment failure which requires innovative new approaches to improve the results of active immunotherapy with vaccine. The treatment failure could result from an immune response that is clinically absent, ineffective, or unfavorable. The last-named possibility is the most intriguing, not only because it mandates an immediate change in therapy but also because it suggests that the vaccine formulation can be manipulated so that its immunogenic antigens elicit a different immune response, particularly a different antibody response.

There are other immunotherapeutic treatment protocols underway in several institutions. One of the trials is conducted by Eastern Co-operative Oncology Group using a ganglioside antigen called GM_2. The rationale for using GM_2 in treatment of melanoma patients is that it is considered to be differentiation antigen of human melanoma. An antigen is considered to be differentiation antigen only when the precursor cell of the tumor does not express the antigen. However the criteria is modified with reference to GM_2 since the precursor cell, the normal melanocytes, express in trace quantities when compared to the tumor cells.

Tumor growth after therapeutic treatments not only depends on the intrinsic characteristics of the tumor, but also is influenced by the host's immune mechanisms. The interaction between tumor and immune system may lead to tumor rejection, regression or elimination. Tumor-derived factors may suppress specific immune responses and downregulate specific antibodies that would otherwise cause tumor rejection. When cancer vaccines augment

antibodies tumor regression rather than tumor growth should be expected, but it is not always the case. Tumor growth after treatment with cancer vaccine could be due to elicitation of an unfavorable immune response by the cancer vaccine. There are two observations supporting such a possibility. One study documents immune-mediated downregulation of tumor-antigen expression, allowing the escape of tumor cells from immune surveillance (Yeh *et al.*, 1981). The other study shows that vaccine-induced IgG response to a 90 kDa glycoprotein melanoma-associated antigen correlated with decreased overall survival. Several hypotheses have been proposed for poor prognosis related to vaccine-induced IgG antibodies, such as unfavorable antigenic modulation (down regulation) leading to failure in immunorecognition (Jones *et al.*, 1996).

One or more of these mechanisms may be involved in tumor growth after immunotherapy with cancer vaccine. Documentation of an immune response promoting tumor progression will be helpful in determining if a particular treatment protocol should be altered. Similar identification of all the important tumor-antigens that elicit immune response leading to tumor regression is important.

Recent observations indicate that the patients who naturally produce antibodies to the tumor associated antigens show improved survival (Jones *et al.*, 1981; Livingston *et al.*, 1994). Furthermore survival of the patients can be improved by inducing and augmenting the level of these antibodies (Jones *et al.*, 1981; Livingston *et al.*, 1994). Methods for inducing high levels of systemic immunity against these tumors have been previously reported (Bystryn *et al.*, 1995; Morton and Ravindranath, 1996; Morton *et al.*, 1992, 1994; Wallack *et al.*, 1995; Livingston *et al.*, 1994). Since antigens are rarely specific to tumors, the neoplastic-differentiation (the antigens that are expressed in association with tumor formation) antigens are considered as ideal targets of cancer immunotherapy. For example, gangliosides GM_2 and GD_2 are considered as differentiation antigens in human melanoma. Murine and human monoclonal antibodies against GM_2 and GD_2 have been associated with tumor regression (Cheung *et al.*, 1988; Reisfeld and Schrappe, 1990 Irie and Morton, 1986). Immunization of melanoma patients with pMCV expressing these antigens (Morton 1992, 1994) or with purified GM_2 conjugated to KLH and an adjuvant QS-21, augmented anti-GM_2 IgM production and improved overall survival (Livingston *et al.*, 1994). As a result, clinical trials of passive and active specific immunotherapy in melanoma are targeting GM_2 (Livingston *et al.*, 1994).

These observations revealed that tumor antigens which are functionally related to tumor differentiation, proliferation, invasion or progression are potential targets for immunotherapy. There is a need to identify functionally important tumor differentiation antigens that are immunogenic in man for the purpose of developing a more potent and effective vaccine to target tumor cells by immunotherapy.

SUMMARY OF THE INVENTION

It is, therefore, a goal of the present invention to provide methods and compositions relating to the treatment of cancer generally, and melanoma specifically. In particular, it is a goal of the present invention to provide vaccines for the active stimulation of immune response in subjects and for passive transfer of immune effectors to subjects. In satisfying these objectives, the following embodiments are provided.

In a first embodiment, there is provided an antigenic composition comprising a Sialyl Lewis antigen and an adjuvant. The Sialyl Lewis antigen may be any Sialyl Lewis antigen but, in a particular embodiment, it is Sialyl Lewis^x or Sialyl Lewis^a. The vaccine may comprise a plurality of Sialyl Lewis antigen species, for example, including both Sialyl Lewis^x and Sialyl Lewis^a. The Sialyl Lewis antigen may be contained in the carbohydrate moiety of a glycoprotein, mucin or glycolipid, for example, of the glycoprotein is CEA or MUC-1. The vaccine may further comprise one or more tumor-associated ganglioside antigen species. The Sialyl Lewis antigen may be a multimer, such as a dimer, a trimer, a tetramer, a pentamer, a hexamer, a septamer, an octamer, a nonamer or a decamer. The Sialyl Lewis antigen is a heteromer. The Sialyl Lewis antigen may be in a soluble form and/or conjugated to said adjuvant.

The adjuvant of the vaccine may be a biopolymer or a biomembrane. The biomembrane may be a bacterial membrane. The bacterial membrane may be derived from a bacterial genus from the group consisting of *Mycobacterium, Salmonella, Escherichia, Heliobacter, Staphylococcus* and *Streptococcus*. The composition also may comprise a bacterial coat, a bacterial polysaccharide, a bacterial glycolipid, bacterial nucleic acid, bacterial lipid or bacterial protein. The adjuvant also may be incomplete Freund's, complete Freund's, bacterial cell wall, KLH, LTA, GTA, Chitin, MDP, threonyl-MDP, MTPPE, BCG, cell wall skeleton, trehalose

dimycolate, QS21, Quil A or lentinen. The adjuvant may be a lipopolysaccharide group adjuvant, for example, a detoxified endotoxin, lipopolysaccharide, lipid A, monophosphoryl lipid A (MPL) or derivatives thereof. The adjuvant may be covalently linked to said antigen.

The composition may further comprise at least a first cell, wherein at least one Sialyl Lewis antigen is disposed on the cell membrane of said cell. Other Sialyl Lewis antigens or non-Sialyl Lewis antigens may be disposed on the cell membrane. The cell may be a human cell, a murine cell, a guinea pig cell or other cell. The cell may be a fibroblast, an erythrocyte, a tumor cell or other cell. The cell may be irradiated. The tumor cell may be a melanoma cell, for example, a M27, M18, M14, M111, M22, M7, M102, M108, M16, M104, M109, M25, M24, M10 or M101 cell. The composition may further comprise at least a second cell that it genetically distinct from said first cell.

The cell is genetically engineered to express a exogenous gene. The exogenous gene may encode a cytokine, a Sialyl Lewis antigen, an adjuvant, glycosylating enzyme or a Sialyl Lewis antigen carrier. The cell may be genetically engineered to overexpress a normal cell product. The normal cell product may be a Sialyl Lewis antigen or a cytokine.

The cell-based composition may comprise a population of cells that includes between about 3 and about 24 x 10^6 and about 24 x 10^8 of BCG per 10^6 cells. The cell based composition may comprise a population of cells that includes between about 24 x 10^6 and about 200 μg of MPL per 10^6 cells.

The composition may further comprise a tumor-associated ganglioside, for example, a cell surface ganglioside, a neural ganglioside or extraneural tissue ganglioside. Examples of gangliosides GD_3, GD_2 or GM_2, GM_3, GM_{1a}, GM_{1b}, GD_{1a}, GD_{1b}, GT_{1a}, GT_{1b}, GT_3 and GQ_{1b}.

The composition may further comprise a liposomal biomembrane, such as a unilamellar, multilamellar and/or cochlear vesicle. The composition may further comprise a biopolymer made of porous polystyrene [poly(styrene-divinylbenzene)] (PSDVB).

In another embodiment, there is provided a vaccine comprising a Sialyl Lewis antigen and an adjuvant in a pharmacologically acceptable buffer, diluent or excipient.

In yet another embodiment, there is provided a method of stimulating an immune response in an animal comprising the steps of (a) providing a pharmacologically acceptable composition comprising a Sialyl Lewis antigen and an adjuvant; and (b) administering said composition to said animal in an amount effective to stimulate an immune response against said

Sialyl Lewis antigen. The Sialyl Lewis antigen may be disposed on the surface of a cell. The cell may be an erythrocyte or a tumor cell. The tumor cell may be irradiated. The tumor cell may be an autologous tumor cell obtained from said animal. The tumor cell may be an allogeneic tumor cell. The composition may be administered by injection, in particular, by injection into a solid tumor site.

In still yet another embodiment, there is provide a method for treating a tumor in an animal comprising the steps of (a) providing a composition comprising a Sialyl Lewis antigen and an adjuvant; and (b) administering said composition to said tumor in an amount effective to inhibit the growth of said tumor.

In still yet another embodiment, there is provided a method for inducing a predominantly IgM response in an animal comprising the steps of (a) providing a pharmacologically acceptable composition comprising a Sialyl Lewis antigen and an adjuvant; and (b) administering said composition to said animal in an amount effective to stimulate an IgM response.

In still yet another embodiment, there is provided a method for screening a composition for the ability to induce an anti-tumor immune response in an animal comprising the steps (a) providing a pharmacologically acceptable composition comprising a Sialyl Lewis antigen and an adjuvant; (b) administering said composition to said animal in an amount effective to stimulate an immune response; and (c) determining the extent of an IgM response in said animal against said Sialyl Lewis antigen.

In still yet another embodiment, there is provided a kit comprising, in suitable container means, a pharmaceutically acceptable composition comprising a Sialyl Lewis antigen and an adjuvant. Another embodiment comprises a human monoclonal antibody that reacts immunologically with a Sialyl Lewis antigen, wherein said monoclonal antibody is of the IgM class.

In still yet a further embodiment, there is provided a method of isolating a Sialyl Lewis specific B cell comprising the steps of (a) administering the vaccine of claim 50 to a subject; and (b) isolating said B cell. The method may further comprise the step of immortalizing said B cell. The method also may further comprise the step of (a) culturing said B cell; and (b) purifying antibodies generated by said B cell.

It is also understood that in the practice of the present invention that any of the compositions of the present invention, for example those comprising Sialyl Lewis antigens, those

comprising a cell or a biomembrane, or any other composition as described herein may also contain other antigenic compositions that are useful in the treatment of tumors or other diseases or conditions. Such antigens would include, but would not be limited to urinary TAA (glycoprotein 90), fetal antigen (glycoprotein 70), 810 peptide (43 dKa), a melanoma associated ganglioside lipoprotein 180, MART-1/Melan A, glycoprotein 75 (gp 75 TRP), glycoprotein 100 (gp 100/pmel 17), high molecular weight melanoma antigen as defined in Morton and Barth, 1996, (incorporated herein by reference), lipoprotein antigen, MAGE-1, MAGE-3, tyrosinase and MUC-1. Any of such antigens would be effective when combined with a composition comprising Sialyl Lewis antigens and an adjuvant, or even in a whole cell vaccine. All such compositions would be encompassed by the spirit and scope of the appended claims.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1A, FIG. 1B, FIG. 1C, FIG. 1D, FIG. 1E and FIG. 1F: Expression of Sialyl Lewis^a (sLe^a) and Sialyl Lewis^x (sLe^x) in the melanoma cell surface as monitored by a novel cell-suspension ELISA using monospecific murine monoclonal antibodies (KM93 IgM and KM231 IgG1) as described in Example 1. The vertical bars represent standard deviation of 5 analysis. **FIG. 1A**. Expression in cells obtained from biopsy specimens of human melanoma. **FIG. 1B**. Expression in human melanoma cell lines grown in the medium (RPMI-1640) containing fetal calf serum (FCS). **FIG. 1C**. Expression in polyvalent melanoma cell vaccine (pMCV) used in phase II and phase III clinical trial. pMCV constitutes the three human melanoma cell lines

grown in the medium (RPMI-1640) containing fetal calf serum (FCS). **FIG. 1D.** Expression in mouse B16 melanoma cell line grown in the medium (RPMI-1640) containing fetal calf serum (FCS). **FIG. 1E.** Identification of carrier molecules of sLe structures in human melanoma cells. **FIG. 1F.** The relative expression of sLe^x, sLe^a, GD_3, and GD_2 in melanoma cell line constituting pMCV do not change after repeated passing the cells in the culture media and cryopreservation.

FIG. 2A, FIG. 2B, FIG. 2C and FIG. 2D: Sialyl $Lewis^x$ and Sialyl $Lewis^a$ are immunogenic in man indicating that they can be potential targets for immunotherapy. **FIG. 2A.** Profile of a responder patient (# B0213) AJCC stage III melanoma. **FIG. 2B.** Profile of a non-responder patient (# B0725) AJCC stage III melanoma. **FIG. 2C.** Profile of a responder patient (# B0815) AJCC stage III melanoma. **FIG. 2D.** Profile of a responder patient (# C0819) AJCC stage III melanoma.

FIG. 3A, FIG 3B and FIG. 3C: Sialyl Lewis antigens induce immune response in the preclinical mice model for melanoma. IgM response correlates with tumor regression and IgG response is associated with tumor growth. **FIG. 3A.** pMCV induces IgG and/or IgM antibody responses to sLe^x in the preclinical model depending on the adjuvant conjugated to pMCV. **FIG. 3B.** pMCV induces IgG and/or IgM antibody responses to sLe^a in the preclinical model depending on the adjuvant conjugated to pMCV. **FIG. 3C.** pMCV induces IgG and/or IgM antibody responses to the ganglioside GM_3 in the preclinical model depending on the adjuvant conjugated to pMCV.

FIG 4A, FIG. 4B, FIG. 4C and FIG. 4D: The nature and titer of antibody response to Sialyl Lewis antigens and the ganglioside GM_3 is correlated with tumor growth. **FIG. 4A.** Correlations between mean anti-sLe^x IgG: IgM titer ratios and tumor growth in mice immunized with different vaccine formulations. **FIG. 4B.** Significant linear relationship between anti-sLe^x IgM titer and tumor growth in mice immunized with different vaccine formulations. **FIG. 4C.** Correlations between mean anti-sLe^a IgG: IgM titer ratios and tumor growth in mice immunized with different vaccine formulations. **FIG. 4D.** Correlations between mean anti-GM_3 IgG: IgM titer ratios and tumor growth in mice immunized with different vaccine formulations.

FIG. 5A and FIG. 5B: The rate of tumor growth in mice varies depending on the vaccine-adjuvant combination. **FIG. 5A.** Tumor progression after immunization with different formulations of pMCV. **FIG. 5B.** Overall survival after challenge of tumor cells (50,000) in different immunization groups.

FIG. 6A, FIG. 6B and FIG. 6C: Cell-surface expression of sLe^a and sLe^x on tumors growing in immunized mice shows selective loss of expression of sLe^x. **FIG. 6A.** Selective loss of expression of Sialyl $Lewis^x$ in tumors grown in immunized mice. **FIG. 6B.** No loss of expression of GM_3 in tumors grown in immunized mice. **FIG. 6C** Expression of Sialyl $Lewis^a$ and GM_3 is seen in spite of the presence of shows surface IgM and IgG on tumor cells obtained from biopsies of immunized mice.

FIG. 7: Relationship between the ratio of IgG : IgM titers and tumor growth.

FIG. 8A, FIG. 8B, FIG. 8C and FIG. 8D: sLe^a and sLe^x are immunogenic in guinea pig model. **FIG. 8A.** Delayed-type hypersensitivity reaction to xenogenic pMCV in the skin of guinea pigs after the fourth immunization with different formulations of pMCV. **FIG. 8B, FIG. 8C and FIG. 8D.** Differences in the anti-sLe IgG antibody response induced by pMCV and the lysate of pMCV in the guinea pig model. After four immunizations, pMCV-BCG induced significantly high antibody response to sLe^x **(FIG. 8B)** and sLe^a **(FIG. 8C).** **FIG. 8D** summarizes the superiority of whole cell vaccine in inducing antibody response to sLe^a and sLe^x.

FIG. 9A and FIG. 9B: IgM antibody titers against sLe antigens depicting post- and pre-immune IgM levels in 10 patients with colorectal carcinoma. Serum anti-sLe^x IgM (FIG. 9A) and anti-sLe^a (FIG. 9B) IgM titers are shown.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

The present invention is designed to take advantage of a surprising immunologic property of a class of molecules designated as Sialyl Lewis antigens. Though these antigens have been correlated with certain malignancies, their role in tumor establishment and disease progression is unclear. The fact that such molecules are found on normal cells (*e.g.*, neutrophils), suggests that they would not be useful for diagnostic or therapeutic purposes. Moreover, because these molecules are expected to be seen as "self" by host immune systems since they are present on normal tissues, one would also suspect that immune responses to Sialyl Lewis antigens would be nonexistent or minimal.

The present inventors have observed first that Sialyl Lewis antigens are expressed on certain malignant cells, whereas the corresponding normal cells do not express these antigens. This provides both a diagnostic and prognostic incentive to determine expression of Sialyl Lewis

antigens on cells where these antigens are not normally expressed. Second, the inventors have found, surprisingly, that certain normal individuals have, in a disease-free state, circulating antibodies to Sialyl Lewis antigens. Other individuals only develop immune responses after developing tumors that express these antigens, or after immunization with a vaccine containing a Sialyl Lewis antigen. Yet another group of individuals is completely unable to respond to Sialyl Lewis antigens. Third, it further has been observed that the relevant immune response is, in fact, an IgM response with only rare IgG responses observed to this antigen.

Thus, it is proposed that individuals capable of responding to Sialyl Lewis antigens may be immunized with a vaccine containing these antigens. This may prove effective where these individuals have a higher chance of developing a cancer, such as melanoma. A more direct and facile approach to treating both these individuals, and individuals that cannot respond, is to transfer antibodies from individuals who produce, in a non-disease state, anti-Sialyl Lewis antibodies (passive immunization). Yet another approach is to isolate B lymphocytes or plasma cells that produce anti-Sialyl Lewis antibodies and harvest human antibodies (monoclonal or polyclonal) *in vitro*. Such antibodies may then be provided to individuals in need thereof.

The inventors propose that for an antigen to be a suitable candidate for cancer vaccine a number of criteria have to be fulfilled. These criteria include that the antigen should be (1) functionally important as indicated by the ability of the antigen to render the cell vulnerable to immune destruction or serve as a target to prevent extravasation and metastases; (2) capable of inducing antitumor antibody response and/or cell mediated immune response; (3) a true tumor-differentiation antigen and (4) a tumorigenic antigen and (5) the antibody response to the antigen should have clinical benefit.

Expression of sLe^x is correlated with increased metastatic potential of tumor cells (Ogawa *et al.*, 1994b; Narita *et al.*, 1993; Nakamori *et al.*, 1993) and poor patient survival (Narita *et al.*, 1993; Nakamori *et al.*, 1993). However, recent reports indicate that sLe^x and a structurally similar sLe^a may be expressed in human melanoma (Kuzendorf *et al.*, 1987; Kageshita *et al.*, 1995).

To improve the effectiveness of vaccine therapy, it is important to identify and modify the factors responsible for treatment failure. The inventors have obtained data from a preclinical model for melanoma immunotherapy which revealed that there is an adverse correlation between

anti-Sialyl Lewis^x (sLe^x) high IgG titer and tumor growth. The results suggested that anti-sLe^x IgG may be associated with factors which enhance tumor growth. In contrast to this it appears that IgM may be associated with tumor regression and improved survival. The ratio of IgG to IgM could accurately predict the clinical outcome of immunotherapy. It was also observed that the tumor growing in immunized mice did not express sLe^x on the tumor cell surface suggesting that immunization had induced antigenic modulation.

In the present application, the inventors demonstrate that human melanoma cells from tumor biopsies and two of the three melanoma cell lines constituting a melanoma cell vaccine (MCV) expressed high levels of sLe^x (Table 4). No sLe^x was found in normal melanocytes of the skin, indicating that sLe^x upregulation occurs during tumorigenesis, and sLe^x is a differentiation antigen in human melanoma. Patients receiving vaccine developed high titers of antibodies to sLe^x indicating that the antigen is immunogenic in man. Because of the putative roles of sLe^x in extravasation, metastasis and tumor progression, the inventors hypothesize that an immunotherapy which elicits an antibody response against sLe^x may have clinical relevance. Stimulation of a humoral response to sLe ligands may generate antibodies with the ability to prevent extravasation, metastasis, and tumor progression, and promote antibody-mediated cytotoxicity.

The inventors further demonstrate that in a murine model for melanoma, immunization of pMCV induced both IgG and IgM antibodies to sLe^x. High IgM response is correlated with tumor regression, whereas high IgG response is associated with tumor growth. The present invention provides for the generation of different subclasses (IgM and IgG isotypes) of monoclonal antibodies to be used in the treatment of melanoma and tumor growth. In this regard the inventors have used specific adjuvants that will yield IgM antibodies. It was discovered that MPL and BCG generate high titer IgM antibodies. These adjuvants may therefore be used in combination with the antigens described herein for development of vaccines for active specific immunotherapy of melanoma and for development of human monoclonal antibodies for passive immunotherapy of melanoma.

The inventors have identified sLe^x as a novel immunogenic melanoma-differentiation antigen and a potential target for immunotherapy in human melanoma. Sialyl Lewis^x is not expressed by normal melanocytes, but is found in neutrophils activated by bacterial invasion.

Furthermore, Sialyl Le^x was found in melanoma tumor biopsies as well as in cultured tumor cells.

Augmenting the antibody response against this antigen may prevent tumor growth and metastasis and the hypothesis was tested in a murine model for melanoma model. The transplanted tumor metastasized in mice that did not produce antibodies to this antigen, but regressed in mice that produced high IgM to the antigen. However, tumor growth is greater in mice that produced high IgG antibodies, and cells from these tumors failed to express the antigen. Thus the two classes of antibody response to sLe^x distinguished favorable from unfavorable responses, and may explain the apparent lack of survival benefit of immune responses seen in a number of clinical trials of vaccine therapy.

The inventors have shown that a high IgM titer and/or low IgG:IgM ratio constitute a favorable response that is associated with delayed tumor progression and prolonged survival in a preclinical model of melanoma immunotherapy. The inventors show that a favorable antibody response can be generated by adjustment of adjuvant formulations of vaccine. Hence the present invention further provides improved compositions and methods for the treatment of melanoma and indeed, other neoplastic states through the generation of novel vaccines. The preparation of compositions for active specific as well as passive immunotherapy using the inventors novel observations are detailed herein below.

1. Sialyl Lewis Antigens

Sialyl $Lewis^x$ (sLe^x: *Siaα2,3Galβ1,4(Fucα1,3)GlcNAcβ1,3Gal*-R) and sialyl $Lewis^a$ (sLe^a : *Siaα2,3Galβ1,3(Fucα1,4)GlcNAcβ1,3Gal*-R) are ligands for the endothelial cell adhesion molecule, E-selectin (Phillips *et al.*, 1990; Walz *et al.*, 1990). These ligands constitute the carbohydrate moieties of tumor-associated gangliosides (when attached to lipids) (Magnani *et al.*, 1982), the human carcinoembryonic antigen family (Anostario *et al.*, 1994), and human pancreatic MUC-1 antigen (when attached to proteins) (Ho *et al.*, 1995), and are identified in carcinomas of the skin (Groves *et al.*, 1993), stomach, pancreas (Takada *et al.*, 1995), lung (Shimizu *et al.*, 1993; Kawai *et al.*, 1993; Ogawa *et al.*, 1994), colon (Nakamori *et al.*, 1993), breast (Narita *et al.*, 1993) and prostate (Martensson *et al.*, 1995). These ligands are expressed on human neutrophils, and bind to E-selectin on activated endothelial cells and promote

extravasation of the neutrophils at sites of inflammation (Phillips *et al.*, 1990; Walz *et al.*, 1990). The same mechanism is implicated in the extravasation and metastasis of human carcinoma cells (Groves *et al.*, 1993; Takada *et al.*, 1995; Shimizu *et al.*, 1993; Kawai *et al.*, 1993; Gangadharam and Pratt, 1983).

Expression of sLe^x is strongly correlated with increased metastatic potential of tumor cells (Groves *et al.*, 1993; Takada *et al.*, 1995a, b) and poor patient survival (Ogawa *et al.*, 1994a; Ogawa *et al.*, 1994b). Early investigators (Magnani *et al.*, 1982; Ohta *et al.*, 1995; Hanai *et al.*, 1986; Kunzendorf *et al.*, 1987; Kageshita *et al.*, 1995) were unable to demonstrate sLe^x and sLe^a structures in human melanoma. However, a recent histopathological report on melanoma patients from Japan suggested that sLe^x and sLe^a are expressed on melanoma cells (Kageshita *et al.*, 1995). In previous investigations, employing Cs-ELISA (Ravindranath *et al.*, 1996), the inventors observed both sLe^x and sLe^a on the surface of human melanoma cells derived from tumor biopsies as well as on those grown *in vitro*. The finding of most relevance to pathophysiology of melanoma is the consistent appearance of sLe on melanoma tumor biopsies. The inventors attribute the success to achieve such consistent results to the sensitivity of the inventors' new cell-suspension ELISA (Ravindranath *et al.*, 1996). The sensitivity of the assay depends not only on the monospecificity of the anti-sLe monoclonal antibodies, but also on the optimal viability (>85%) and density of cells required for the assay (Ravindranath *et al.*, 1996). In the present invention, the inventors demonstrate that all of the biopsy specimens analyzed expressed sLe^x and sLe^a, with the expression of sLe^x being more dominant.

The inventors suggest that the persistent expression of sLe structures in melanoma cells grown both *in vivo* and *in vitro* (even after several passages in tissue culture), and the absence of these antigens in 10% FBS used in cell culture suggest that sLe antigens expressed on the surface of melanoma cells are not of exogenous origin. $SialylLe^x$ is absent and sLe^a minimally expressed on the surface of normal human melanocytes (early passages of cells grown in a serum-free medium). The inventors studies described herein demonstrate that there is a limited expression of these antigens in melanocytes and an enhanced expression after neoplastic transformation. These observations show that the sLe antigens of the present invention are indeed differentiation antigens in human melanoma. Expression of sLe^x is analogous to melanoma-associated ganglioside GD_2, which is expressed on tumor cells but not detected on normal melanocytes (Morton *et al.*, 1994).

In clinical immunotherapy, tumor-associated carbohydrate antigens on the cell surface are targeted by monoclonal antibodies (Irie and Morton, 1989; Vadhan-Raj *et al.*, 1986; Reisfeld and Schrappe, 1990) (passive immunotherapy) or by cancer vaccines (Morton *et al.*, 1994; Livingston, 1995; Ravindranath and Morton, 1996; Wallack *et al.*, 1995) (active specific immunotherapy). In the present invention, it is demonstrated that sLe^x and sLe^a expressed on the surface of the inventors' melanoma vaccine induce antibody responses in melanoma patients. The titers of anti-sLe^x and anti-sLe^a IgM antibodies are low (anti-sLe^a IgM [Mean $\pm$ SE]: 140 $\pm$ 17, n = 18; anti-sLe^x IgM: 267 $\pm$ 40, n = 20) in normal healthy donors. However the preimmune level of the antibodies in melanoma patients (anti-sLe^a IgM [Mean $\pm$ SE]: 643 $\pm$ 144, n = 13; anti-sLe^x IgM: 548 $\pm$ 134, n = 13) were elevated two to four fold higher than that observed in normal volunteers (Table 6). This difference suggests that anti-sLe antibodies are natural autoantibodies and the antigens derived from tumor tissues can naturally augment the production of antibody to facilitate clearance of tumor derived antigens. After immunization with pMCV, the mean level of antibodies increased further (anti-sLe^a IgM [Mean $\pm$ SE]: 1164 $\pm$ 330, n = 13, $p < 0.01$; anti-sLe^x IgM: 1365 $\pm$ 456, n = 13, $p < 0.05$).

The present invention clearly shows that in stage III melanoma patients, pMCV-BCG induced a twofold or greater increase in IgM antibody titers against sLe^a and sLe^x suggesting that immunization with pMCV-BCG may further augment the production of the natural anti-sLe autoantibodies. The percentage of patients responding to sLe antigens in pMCV was greater than the percentage responding to GD_3, GD_2, GM_3 in pMCV (Table 1).

TABLE 1

Antibody response to Sialyl Le antigens and melanoma-associated gangliosides in AJCC Stage III Melanoma patients after immunization with pMCV.

Antibodies*	Number of patients	Number of responders	Percentage of responders
Anti-GM_3 IgM**	12	2	17
Anti-GM_2 IgM**	12	8	67
Anti-GD_3 IgM**	12	4	33
Anti-GD_2 IgM**	12	3	25
Anti-sLe^a IgM	12	5	42
Anti-sLe^x IgM	12	5	42

*Antibody response to sLe antigen is measured as titers in ELISA, whereas antiganglioside antibody response was measured as absorbency after dilution of sera (1/1000) in a single point quantitation ELISA. Patients with a twofold or greater increase in IgM levels from the preimmune level were considered responders.

**Data from Morton *et al.*, 1994.

The data described in the present invention for the first time, demonstrate the immunogenicity of sLe^x and sLe^a in humans. Indeed, pMCV-BCG induced high titers of IgM to both protein-associated sLe^x and lipid-associated sLe^a. Development of a peak antibody response after two immunizations suggests that pMCV-BCG induced antibody response against sLe^x and/or sLe^a. Using BCG as antigen in ELISA, the inventors have tested for the presence of sLe^x and sLe^a in the mycobacterium. The inventors' results showed that BCG does not contain the sialyl antigens. Therefore, the inventors infer that BCG served as an immunostimulant in augmenting antibodies to sLe antigens expressed by pMCV.

Anti-sLe IgM antibodies augmented by melanoma vaccine may also be involved in clearance of shed antigens which may be immunosuppressive (Fukushi *et al.*, 1985) in addition to involving in complement and phagocyte (neutrophils and macrophages)-mediated antibody dependent cytotoxic mechanisms. These anti-sLe IgM could potentially opsonize the tumor cells to prevent their extravasation, intravasation, and metastatic potential. *In vitro* studies document

that the binding and extravasation of neutrophils as well as cancer cells can be arrested by coating the cells with anti-sLe^x monoclonal antibodies (Groves *et al.*, 1993; Kawai *et al.*, 1993; Gangadharam and Pratt, 1983). Using a murine model, the inventors have observed that mice with a poor anti-sLe antibody response to immunization with pMCV- KLH developed lung metastases when challenged with syngenic melanoma cells expressing sLe^a and sLe^x. In contrast, challenge tumor cells failed to metastasize in immunized mice (MCV-BCG) that had developed high titers of anti-sLe antibodies to sLe antigens.

From the foregoing discussion, a number of important criteria suggest that sLe^x and sLe^a are potential targets for immunotherapy in human melanoma and other neoplasms containing these antigens. In the first instance their specific upregulation (density of expression) on tumor cells; secondly, their immunogenicity in melanoma patients; thirdly their function as differentiation antigens of human melanoma; and fourthly their role as E-selectin ligands.

The present invention clearly establishes sLe antigens as melanoma differentiation antigens which are immunogenic in patients immunized with pMCV. This enables the inventors to describe the prognostic relevance of anti-sLe antibodies and develop strategies to augment anti-sLe antibody response. The B lymphocytes from the inventors' vaccine recipients can be excellent source for developing human monoclonal antibodies to sLe antigens for passive immunotherapy of melanoma. Furthermore the inventors contemplate that these antigens will also be useful in the preparation of vaccine for use in active specific immunotherapy. The preparation and use of such compositions is described in further detail herein below.

The inventors have identified favorable and unfavorable antibody responses against the sLe antigens described herein. The inventors have demonstrated that vaccines that produce a high IgM titer correlate well with tumor regression. Hence in a preferred aspect the sLe antigens of the present invention will be combined with particular adjuvants identified by the inventors as producing favorable high titer of IgM antibodies and low titer of IgG antibodies. The vaccines and antibodies against the sLe antigens of the present invention may be used alone or in combination with other conventional vaccination and cancer treatment strategies. The vaccines may further be administered in combination with vaccines against other tumor specific antigens as described herein below.

2. Components of the Vaccine

The inventors have documented, for the first time, in this application that Sialyl Le^x and Sialyl Le^a are immunogenic antigens (similar to GM_2, an immunogenic carbohydrate antigen currently under study by Eastern Cooperative Oncology Group (ECOG trial) and that one of these antigens, sLe^x, is a differentiation or tumorigenic antigen. Furthermore, both sLe^x and sLe^a are over-expressed tumor antigens. Other investigators have documented that sLe antigens are functionally important antigens in extravasation and metastasis and that sLe antigens are expressed in other human cancers. Based on these above characteristics, the inventors record in this application that sLe antigens are unique and most important melanoma-associated antigens and potential targets for both passive and active specific immunotherapy of melanoma and other cancers sharing these antigens. As such the inventors contemplate the production of vaccines and other compositions for use in immunotherapeutic applications. Vaccines comprising the sLe antigens may further comprise other components such as other melanoma antigens, adjuvants, and cellular components and the like, these components are discussed in further detail herein below, to be used in combination with the present invention.

A. Other Melanoma Associated Carbohydrate Antigens

The gangliosides GD_3, GD_2 and GM_2 are important human melanoma-associated antigens (Ravindranath and Irie, 1988; Ravindranath and Morton, 1991) which do not require T-cell help for immune recognition (Livingston, 1995). These gangliosides are, therefore, important constituents of whole cell (Morton *et al.*, 1994) or lysate vaccines (Wallack *et al.*, 1995). A recently developed cell-suspension ELISA quantifies the cell-surface expression of these major melanoma-associated gangliosides. Cell viability and antigen density on the cell surface are important factors in determining the magnitude of immune response to the antigens.

The ganglioside patterns found in various melanoma tumor biopsies and cell lines, both diversity and uniformity have been observed (Ravindranath *et al.*, 1989). The diversity pertains to the five major gangliosides, namely, GM_3, GM_2, GD_3, GD_2 and O-acetyl GD_3. The parent ganglioside appears to be GM_3; GM_2 and GD_3 arise directly from this ganglioside; and other gangliosides are the products of GD_3. It is believed that an enzyme cascade is involved in ganglioside biosynthesis and that genes responsible for these enzymes may be governing their

diversity (Ravindranath *et al.*, 1989). Tumors found in some patients express more than 90% of GD_3. The uniformity in the preponderance of GD_3 in all tumors indicates that therapy targeting GD_3 in combination with the sLe antigens of the present invention is likely to be an effective treatment for human melanoma as well as a number of other carcinoma.

Melanoma-associated gangliosides have substantial clinical relevance in view of the property of shedding from tumor cells into the circulation. Observations made on the sera of neuroblastoma patients reveal that there is a significant correlation between tumor-associated ganglioside (GD_2) level and clinical stages of the disease, indicating that tumor burden could be a factor involved in shedding of tumor-associated gangliosides (Ladisch *et al.*, 1987). It is also believed that the level of circulating gangliosides correlates with the clinical stages of melanoma (Portoukalian, 1978), and that the serum levels of melanoma-associated gangliosides will be an effective diagnostic indicator of the different stages of melanoma.

Melanoma-associated gangliosides have also been found in other malignant human tissues. This heightens the usefulness of both the diagnostic embodiments of the invention, and the aspects of the invention that concern generating an effective immune response against ganglioside antigens. The distribution of melanoma-associated gangliosides in malignant human tissues have been extensively reported in a variety of carcinoma including melanoma, glioblastoma, astrocytoma, neurofibrosarcoma, leukemia, thyroid cancer, lung carcinoma and meningia (Carubai *et al.*, 1984; Liepkalns *et al.*, 1981; Berra et al 1983 and 1985; Kyogashima *et al.*, 1987; Bouchon *et al.*, 1985; Tsuchida *et al.*, 1984; Hirabayashi *et al.*, 1987; Fredman *et al.*, 1986; Siddiqui *et al.*, 1984; Miyake *et al.*, 1988; Goff *et al.*, 1983; Irie et al,. 1982; Ravindranath *et al.*, 1988).

B. Adjuvants

Immunization protocols have used adjuvants to stimulate responses for many years. Some adjuvants affect the way in which antigens are presented. For example, the immune response is increased when protein antigens are precipitated by alum. Emulsification of antigens also prolongs the duration of antigen presentation. Other adjuvants, for example, certain organic molecules obtained from bacteria, act on the host rather than on the antigen. An example is muramyl dipeptide (N-acetylmuramyl-L-alanyl-D-isoglutamine [MDP]), a bacterial peptidoglycan. The effects of MDP, as with most adjuvants, are not fully understood. MDP

stimulates macrophages but also appears to stimulate B cells directly. The effects of adjuvants, therefore, are not antigen-specific. If they are administered together with a purified antigen, however, they can be used to selectively promote the response to the antigen.

Adjuvants have been used experimentally to promote a generalized increase in immunity against unknown antigens (*e.g.*, U.S. Patent 4,877,611). This has been attempted particularly in the treatment of cancer. For many cancers, there is compelling evidence that the immune system participates in host defense against the tumor cells, but only a fraction of the likely total number of tumor-specific antigens are believed to have been identified to date. However, using the present invention, the inclusion of a suitable adjuvant into the membrane of an irradiated tumor cell will likely increase the anti-tumor response irrespective of the molecular identification of the prominent antigens. This is a particularly important and time-saving feature of the invention.

The present invention contemplates that a variety of adjuvants may be employed in the membranes of cells, such as tumor cells, resulting in an improved immunogenic composition. The only requirement is, generally, that the adjuvant be capable of incorporation into, physical association with, or conjugation to, the cell membrane of the cell in question.

Those of skill in the art will know the different kinds of adjuvants that can be conjugated to cellular vaccines in accordance with this invention and these include alkyl lysophosphilipids (ALP); BCG; and biotin (including biotinylated derivatives) among others. Certain adjuvants particularly contemplated for use are the teichoic acids from Gram -ve cells. These include the lipoteichoic acids (LTA), ribitol teichoic acids (RTA) and glycerol teichoic acid (GTA). Active forms of their synthetic counterparts may also be employed in connection with the invention (Takada *et al.*, 1995a).

Hemocyanins and hemoerythrins may also be used in the invention. The use of hemocyanin from keyhole limpet (KLH) is particularly preferred, although other molluscan and arthropod hemocyanins and hemoerythrins may be employed.

Various polysaccharide adjuvants may also be used. For example, Yin *et al.* (1989) describe the use of various pneumococcal polysaccharide adjuvants on the antibody responses of mice. The doses that produce optimal responses, or that otherwise do not produce suppression, as indicated in Yin *et al.* (1989) should be employed. Polyamine varieties of polysaccharides are particularly preferred, such as chitin and chitosan, including deacetylated chitin.

A further preferred group of adjuvants are the muramyl dipeptide (MDP, N-acetylmuramyl-L-alanyl-D-isoglutamine) group of bacterial peptidoglycans. Derivatives of muramyl dipeptide, such as the amino acid derivative threonyl-MDP, and the fatty acid derivative MTPPE, are also contemplated.

U.S. Patent 4,950,645 describes a lipophilic disaccharide-tripeptide derivative of muramyl dipeptide which is proposed for use in artificial liposomes formed from phosphatidyl choline and phosphatidyl glycerol. It is said to be effective in activating human monocytes and destroying tumor cells, but is non-toxic in generally high doses. The compounds of U.S. Patent 4,950,645 and PCT Patent Application WO 91/16347, which have not previously been suggested for use with cellular carriers, are now proposed for use in the present invention.

BCG and BCG-cell wall skeleton (CWS) may also be used as adjuvants in the invention, with or without trehalose dimycolate. Trehalose dimycolate may be used itself. Azuma *et al.* (1988) show that trehalose dimycolate administration correlates with augmented resistance to influenza virus infection in mice. Trehalose dimycolate may be prepared as described in U.S. Patent 4,579,945

Amphipathic and surface active agents, *e.g.*, saponin and derivatives such as QS21 (Cambridge Biotech), form yet another group of preferred adjuvants for use with the immunogens of the present invention. Nonionic block copolymer surfactants (Rabinovich *et al.*, 1994; Hunter *et al.*, 1991) may also be employed. Oligonucleotides, as described by Yamamoto *et al.* (1988) are another useful group of adjuvants. Quil A and lentinen complete the currently preferred list of adjuvants. Although each of the agents, and the endotoxins described below, are well-known as adjuvants, these compounds have not been previously incorporated into the membrane of a target cell, as shown herein.

One group of adjuvants particularly preferred for use in the invention are the detoxified endotoxins, such as the refined detoxified endotoxin of U.S. Patent 4,866,034. These refined detoxified endotoxins are effective in producing adjuvant responses in mammals.

The detoxified endotoxins may be combined with other adjuvants to prepare multi-adjuvant-incorporated cells. Combination of detoxified endotoxins with trehalose dimycolate is contemplated, as described in U.S. Patent 4,435,386. Combinations of detoxified endotoxins with trehalose dimycolate and endotoxic glycolipids is also contemplated (U.S. Patent

4,505,899), as is combination of detoxified endotoxins with cell wall skeleton (CWS) or CWS and trehalose dimycolate, as described in U.S. Patents 4,436,727, 4,436,728 and 4,505,900. Combinations of just CWS and trehalose dimycolate, without detoxified endotoxins, is also envisioned to be useful, as described in U.S. Patent 4,520,019.

MPL is currently one preferred immunopotentiating agent for use in improved adjuvant-incorporated tumor cell compositions and vaccines. References that concern the uses of MPL include Tomai *et al.* (1987), Chen *et al.* (1991) and Garg & Subbarao (1992), that each concern certain roles of MPL in the reactions of aging mice; Elliott *et al.* (1991), that concerns the D-galactosamine loaded mouse and its enhanced sensitivity to lipopolysaccharide and MPL; Chase *et al.* (1986), that relates to bacterial infections; and Masihi *et al.* (1988), that describes the effects of MPL and endotoxin on resistance of mice to *Toxoplasma gondii*. Fitzgerald (1991) also reported on the use of MPL to up-regulate the immunogenicity of a syphilis vaccine and to confer significant protection against challenge infection in rabbits.

Thus MPL is known to be safe for use, as shown in the above model systems. Phase I clinical trials have also shown MPL to be safe for use (Vosika *et al.*, 1984). Indeed, 100 $\mu g/m^2$ is known to be safe for human use, even on an outpatient basis (Vosika *et al.*, 1984).

MPL generally induces polyclonal B cell activation (Baker *et al.*, 1994), and has been shown to augment antibody production in many systems, for example, in immunologically immature mice (Baker *et al.*, 1988a; Baker *et al.*, 1988b); in aging mice (Tomai & Johnson, 1989); and in nude and Xid mice (Madonna & Vogel, 1986; Myers *et al.*, 1995). Antibody production has been shown against erythrocytes (Hraba *et al.*, 1993); T cell dependent and independent antigens; Pnu-immune vaccine (Garg & Subbarao, 1992); isolated tumor-associated antigens (U.S. Patent 4,877,611); against syngenic tumor cells (Livingston *et al.*, 1985; Examples 1-5; Ravindranath *et al.*, 1994a; 1994b); and against tumor-associated gangliosides (Ravindranath *et al.*, 1994a;b).

Another useful attribute of MPL is that is augments IgM responses, as shown by Baker *et al.* (1988a), who describe the ability of MPL to increase antibody responses in young mice. This is a particularly useful feature of an adjuvant for use in certain embodiments of the present invention. Myers *et al.* (1995) recently reported on the ability of MPL to induce IgM antibodies, by virtue T-cell-independent antibody production.

Synthetic MPLs form a particularly preferred group of antigens. For example, Brade *et al.* (1993) described an artificial glycoconjugate containing the bisphosphorylated glucosamine disaccharide backbone of lipid A that binds to anti-Lipid A MAbs. This is one candidate for use in certain aspects of the invention.

The MPL derivatives described in U.S. Patent 4,987,237 are particularly contemplated for use in the present invention. U.S. Patent 4,987,237 describes MPL derivatives that contain one or more free groups, such as amines, on a side chain attached to the primary hydroxyl groups of the monophosphoryl lipid A nucleus through an ester group. The derivatives provide a convenient method for coupling the lipid A through coupling agents to various biologically active materials. The immunostimulant properties of lipid A are maintained. All MPL derivatives in accordance with U.S. Patent 4,987,237 are envisioned for use in the MPL adjuvant-incorporated cells of this invention.

Various adjuvants, even those that are not commonly used in humans, may still be employed in animals, where, for example, one desires to raise antibodies or to subsequently obtain activated T cells. The toxicity or other adverse effects that may result from either the adjuvant or the cells, *e.g.*, as may occur using non-irradiated tumor cells, is irrelevant in such circumstances.

C. Cellular Components of Vaccines

a. Natural

A wide variety of cells are also contemplated for use as the cellular components of adjuvant-incorporated cell constructs in accordance with the present invention. Any cell that has a membrane component against which one desires to generate an antibody may be used.

All cell types are thus included so long as, generally, the cell may be isolated in a form with a substantially intact membrane. This includes embryonic cells fibroblasts and non naturally-occurring cells in the context of, *e.g.*, virally-infected cells that express viral protein components on the surface.

i. Melanoma Cells

The melanoma cell vaccine referred to as "MCV", developed by one of the present inventors, may be employed in the present invention. This vaccine consists of three allogeneic

melanoma cell lines that are known to contain effective concentrations of six melanoma associated antigens (MAA). These MAAs have been demonstrated to be immunogenic in melanoma patients. The MAAs include three gangliosides, GD2, GM2 and O-acetyl GD3; and three protein antigens, the lipoprotein M-TAA, and the two glycoproteins M-fetal antigen and M-urinary antigen.

The three human melanoma cell lines used in pMCV are M10, M24, and M101, which were selected from a series of melanoma cell lines after careful examination for the high expression of MAA immunogenic in melanoma patients. These pMCV cells are grown and prepared for administration as described in Morton *et al.* (1993) and in U.S. Patent Application Serial No. 07/961/786, filed October 15, 1992, incorporated herein by reference.

Other melanoma cell lines that may be used include M27, M18, M14, M111, M22, M7, M102, M108, M16, M104, M109, M25, and even M112, M21 and M15 (Tsuchida *et al.*, 1989). Still further melanoma cell lines, established and characterized at the John Wayne Cancer Institute (JWCI), include M12, Mke, Mst, Mmu, Mka, and Mkn. These may also be employed in the present invention. Preferred cell lines will generally be selected from those described herein and by Morton *et al.* (1992; 1993).

Furthermore, the qualitative and quantitative pattern of ganglioside antigen expressed by cell lines, may be modified, by adjusting the culture conditions. Tsuchida *et al.* (1987; 1989) studied the pattern of gangliosides in human melanoma tumor cells, directly obtained after surgery, after growing them in culture and after implanting them in immuno-deficient mice (called nude mice). The ganglioside pattern changed after growing the tumor cells in culture. Interestingly, the pattern reverts back to the original tumor condition after implanting and growing into nude mice. Re-establishment of the original ganglioside patterns after growing in nude mice demonstrates that *in vivo* expression of gangliosides on tumor cell surface is conserved and stable, whereas growing them in culture conditions may alter their synthesis and expression. This fact is important for developing cell lines for vaccine immunotherapy.

The MAA antigens of the pMCV, developed by one of the present inventors, are located on the cell surface, and antibodies to them have been shown to bind with complement and kill melanoma cells *in vitro* (Sidell *et al.*, 1979; Irie *et al.*, 1989;). Immunization of patients with pMCV containing these antigens induces specific immune responses to the MAA (Ravindranath *et al.*, 1989; Euhus *et al.*, 1989). The presence of antibodies to those MAA in melanoma patients

who were not treated with the vaccine was found to correlate with survival, indicating that these MAAs are important in the natural history of melanoma and in modulating the host protective immune responses against this disease (Jones *et al.*, 1981).

As the antigens in the pMCV are present at the cell surface, and as antibodies to MAA both kill melanoma cells and correlate with survival, the pMCV is an ideal vehicle for use in the present invention. Furthermore, as the pMCV vaccine has been used in Phase II clinical trials, it has already been proven to be safe for human administration. Indeed, patients receiving this vaccine have survived significantly longer than patients previously treated with other regimens of immunotherapy or chemotherapy and, when compared to previous trials, pMCV was found to be significantly more effective in eliciting specific humoral and cell-mediated immune responses.

As discussed herein, pMCV containing adjuvants in the cell membrane will also be useful for active immunotherapy in other types of human cancer, since five of the six tumor associated antigens found in the pMCV compositions are also present in other types of human neoplasms. The lipoprotein antigen (180 kD) is the only one whose distribution is restricted to melanoma that has not, at the present time, been shown to induce antibodies that cross-react with other types of human neoplasms.

The sources of a variety of other melanoma cells that may be used in this invention are known. For example, U.S. Patents 5,194,384 and 5,030,621, each incorporated herein by reference, concern methods for preparing cell-free vaccines that require the culture of human cancer cells. The same cells could be employed in this invention.

In the case of melanoma, it is believed that those patients who respond well to active immunotherapy have a high level of anti-ganglioside IgM that acts to clear the shed gangliosides. In contrast, in non- or poor-responders, the level of the tumor derived gangliosides is believed to be too high, or the level of anti-ganglioside antibodies too low, resulting in failure to reduce or eliminate the shed gangliosides.

In the treatment of melanoma, the adjuvant-incorporated cell tumor cell compositions disclosed herein would function to augment antibody production and then, as a sequel to the formation of antibodies, the shed gangliosides would be cleared from circulation and immunocompetence restored. The combined adjuvant-incorporated tumor cell formulation thus

has a dual and perpetuating role, *i.e.*, attacking tumor cells by way of antibody binding to their surface antigens, and removing shed antigens from the blood to prevent competition for antibody binding.

ii. Erythrocytes

One type of cells that is particularly suited for use in this invention is the erythrocyte. In terms of clinical treatment for various diseases, particularly in cancer treatment, patients' autologous erythrocytes are contemplated for use. There are many advantages to the uses of erythrocytes, as described in the following section.

A growing tumor sheds it antigens into the circulation. Often these circulating antigens are entrapped by erythrocytes, forming "coated erythrocytes". Portukalian *et al.* (1978) observed that the erythrocytes of melanoma patients obtained before surgical resection of tumor contain large amounts of tumor derived gangliosides. The level of these gangliosides on erythrocytes declined after surgery suggesting that the gangliosides on tumor cell surface reflect the tumor burden and shed gangliosides (Portukalian *et al.*, 1978).

It is a concept of this invention that erythrocytes from cancer patients will likely accommodate many tumor associated antigens. In any event, the erythrocytes of cancer patients are different from that of normal individuals in that they have at least some tumor associated antigens on their surface. The use of "autologous erythrocytes" for adjuvant conjugation is presently envisioned. In this aspect of the invention, erythrocytes would be obtained from a cancer patient, incorporated with adjuvant as described herein, and then administered back into the same patient such that the adjuvant augments the immune response against the tumor associated antigens found on erythrocyte cell surface.

This aspect of the invention has many favorable features. For example, erythrocytes are very abundant cells and are readily obtained. Indeed, it is one of the simplest clinical procedures to obtain a sample of a patient's blood and to prepare the erythrocyte-containing fraction. In contrast, obtaining autologous tumor cells may be difficult due to tissue biopsy or extraction. Autologous tumor cells may also be in scant supply, and can be difficult to grow in sufficient quantities in culture conditions.

In the erythrocyte aspects of the invention there is no need to grow erythrocytes in culture conditions at all. This provides advantages of both time and cost over using autologous or

allogenic tumor cell lines. Furthermore, the erythrocytes are not exposed to artificial culture conditions or to animal serum proteins, such as fetal calf serum, as are cultured tumor cells. This lessens the possibility that the tumor cells exhibit phenotypic changes during cell culture, which may render their antigenic profile one step removed from that of the tumor cells in the animal.

The fact that erythrocytes are anucleated cells provides further advantages. For example, they cannot further mutate when re-administered. Also, the life time of the erythrocyte is finite, which provides for ready control of the adjuvant-incorporated cells circulating in the patient at any given time. Furthermore, unwanted immune responses directed against the nucleus, nuclear proteins, nucleic acids (such as anti-DNA antibodies and anti-nuclear antibodies) can be avoided by using anuclear cells as a therapeutic. As a sequel, the pressure on the immune system and functions will be minimized.

In the practice of the invention, if it was desired to increase the coating of autologous erythrocytes with tumor antigens, this could be readily achieved by adding a sufficient amount of the desired or selected tumor antigens *in vitro*, along with the adjuvant to facilitate immunopotentiation. Also, different antigens, or a combination of antigens, could be added, as desired.

To increase the induction of immune responses that are restricted to the specific tumor-associated antigen coating the erythrocytes, tolerance may be first induced to autologous erythrocytes using tumor-antigen-free erythrocytes. Subsequent exposure to adjuvant-incorporated erythrocytes with tumor antigens would then elicit a specific anti-tumor response. This would likely circumvent any autoimmunity to self-antigens or hemolysis that may possibly occur in this process.

iii. Tumor Cells

A further particular group of cells that are suited for use in this invention are tumor cells. The range of animal and human tumor-derived or associated cells that may be used to stimulate an immune response against a cell component is virtually limitless.

By way of example only many tumor cell lines and their sources are disclosed in the ATCC Catalogues and the skilled artisan is referred to such a cell depository for tumor cell lines that are readily available. Furthermore, analysis of the scientific literature will readily reveal an appropriate choice of cell for any general type desired to be used.

Where an anti-tumor response is desired using the methods of the invention, there is no actual requirement for unmodified tumor cells themselves to be used. Rather, cells that have been modified or engineered to contain membrane accessible tumor antigens may be employed. Such cells may be of virtually any origin, so long as they have been manipulated to express one or more tumor antigens. Expression of tumor antigens can also be achieved by virtually any method, such as, *e.g.*, by admixing the antigens or proteins into the membrane; by fusing tumor cell membrane preparations with the cells; by fusing liposomes containing tumor antigens with the cells; by expressing a recombinant DNA segment encoding a tumor antigen in a cell, and the like.

These techniques allow for one or more cells, or a cocktail of cells, to be prepared and their tumor antigen content generally controlled. Tumor antigens can thus be expressed, or over-expressed as predominant antigens, in cell types that do not normally contain such cells. Additional tumor antigens can also be added to an existing tumor cell, or a previously engineered cell, to provide a "multi-antigen cell".

Many tumor antigens are known in the art that can be utilized in this way. For example, the antigen termed TAG 72 and the HER-2 proto-oncogene protein are selectively found on the surfaces of many breast, lung and colorectal cancers (Thor *et al.*, 1986; Colcher *et al.*, 1987; Shepard *et al.*, 1991). The milk mucin core protein and human milk fat globule, as recognized by the antibodies MOv18 and OV-TL3 (Miotti *et al.*, 1985; Burchell *et al.*, 1983); and the high M_r melanoma antigens that bind to the antibody 9.2.27 (Reisfeld *et al.*, 1982) are further examples.

iv. Normal Fibroblasts Incorporated with sLe antigens

Incorporation of sLe antigens on to the cell surface of fibroblasts or erythrocytes can be done as described by the inventors (Ravindranath *et al.* 1996). Sialyl Lewis antigens in glycolipid form solubilized in ethanol are added to polypropylene tubes and evaporated to dryness in vacuum. After drying, 200 µl of RPMI is added, and tubes are vortexed (2 min.) and sonicated (15 min.) in three cycles. To each tube, 50 µl of 10^6 human skin fibroblasts (clonetics) can be added and incubated for 37°C for 1 hr. After three washings with PSB-HSA, murine anti-sLe monoclonal antibody are added and cells incubated at 7.4°C for 1 hr. For controls, cells are added to ethanol-treated tubes. The controls are divided into two groups: Group 1 (background)

are treated in the same way as glycolipid-incorporated cells; group II (blank) are treated only with the peroxidase-conjugated second antibody. All experimental values are adjusted to the background to obtain information regarding the quantity of sLe antigens incorporated on to the cells.

b. Artificial Membranes

The sLe antigens of the present invention may be incorporated into artificial membranes for eliciting an immune response, such membranes will include liposomal formulations porous beads as described herein below.

i. Liposomes

It previously has been shown that attaching lipopolysaccharide (LPS) and lipid A to synthetic membranes (liposomes) can result in the generation of an immune response to membrane components. This has been proposed to be connected with macrophage recruitment (Verma *et al.*, 1992). U.S. Patent 4,950,645 describes a lipophilic disaccharide-tripeptide derivative of muramyl dipeptide for use in artificial liposomes that is effective in activating human monocytes and destroying tumor cells, but is non-toxic in generally high doses. Thus it is clear that liposomes may be employed to augment an antigenic response. The present invention contemplates the use of liposomes in combination with sLe antigens to produce such a response.

The term liposome is meant to include a wide variety of lipid based vesicles for the incorporation, encapsulation or entrapment of antigens and pharmaceuticals. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991).

"Liposome" is a generic term encompassing a variety of single and multilamellar lipid vehicles formed by the generation of enclosed lipid bilayers. Phospholipids are used for preparing the liposomes according to the present invention and can carry a net positive charge, a net negative charge or are neutral. Dicetyl phosphate can be employed to confer a negative charge on the liposomes, and stearylamine can be used to confer a positive charge on the liposomes. According

to the present invention, sLe antigens are to be encapsulated in a liposome to elicit a favorable immunotherapeutic response.

The physical characteristics of liposomes depend on pH, ionic strength and the presence of divalent cations. Liposomes can show low permeability to ionic and polar substances, but at elevated temperatures undergo a phase transition which markedly alters their permeability. The phase transition involves a change from a closely packed, ordered structure, known as the gel state, to a loosely packed, less-ordered structure, known as the fluid state. This occurs at a characteristic phase-transition temperature and results in an increase in permeability to ions, sugars and drugs.

In addition to temperature, exposure to proteins can alter the permeability of liposomes. Certain soluble proteins deform and penetrate the bilayer, thereby causing changes in permeability. The effects of proteins on the nature of liposomes may be tested and the formulations adjusted to obtain optimal preparations. It is contemplated that the most useful liposome formations for use with the present invention will contain phosphatidylcholine and phosphatidylserine. In other circumstances cholesterol may also be used in producing liposomes.

The ability to trap solutes varies between different types of liposomes. For example, multilamellar vesicles (MLVs) are moderately efficient at trapping solutes, but small unilamellar vesicles (SUVs) are inefficient. SUVs offer the advantage of homogeneity and reproducibility in size distribution, however, and a compromise between size and trapping efficiency is offered by large unilamellar vesicles (LUVs). These are prepared by ether evaporation and are three to four times more efficient at solute entrapment than MLVs. For the present invention, MLVs are preferred.

In addition to liposome characteristics, an important determinant in entrapping compounds is the physicochemical properties of the compound itself. Polar compounds are trapped in the aqueous spaces and nonpolar compounds bind to the lipid bilayer of the vesicle. Polar compounds are released through permeation or when the bilayer is broken, but nonpolar compounds remain affiliated with the bilayer unless it is disrupted by temperature or exposure to lipoproteins. Both types show maximum efflux rates at the phase transition temperature.

Lipids suitable for use according to the present invention can be obtained from commercial sources. For example, dimyristyl phosphatidylcholine ("DMPC") can be obtained from Sigma

Chemical Co., dicetyl phosphate ("DCP") is obtained from K & K Laboratories (Plainview, NY); cholesterol ("Chol") is obtained from Calbiochem-Behring; dimyristyl phosphatidylglycerol ("DMPG") and other lipids may be obtained from Avanti Polar Lipids, Inc. (Birmingham, Ala.). Stock solutions of lipids in chloroform or chloroform/methanol can be stored at about -20°C. Preferably, chloroform is used as the only solvent since it is more readily evaporated than methanol.

Phospholipids from natural sources, such as egg or soybean phosphatidylcholine, brain phosphatidic acid, brain or plant phosphatidylinositol, heart cardiolipin and plant or bacterial phosphatidylethanolamine are preferably not used as the primary phosphatide, *i.e.*, constituting 50% or more of the total phosphatide composition, because of the instability and leakiness of the resulting liposomes.

Liposomes used according to the present invention can be made by different methods. The size of the liposomes varies depending on the method of synthesis. A liposome suspended in an aqueous solution is generally in the shape of a spherical vesicle, having one or more concentric layers of lipid bilayer molecules. Each layer consists of a parallel array of molecules represented by the formula XY, wherein X is a hydrophilic moiety and Y is a hydrophobic moiety. In aqueous suspension, the concentric layers are arranged such that the hydrophilic moieties tend to remain in contact with an aqueous phase and the hydrophobic regions tend to self-associate. For example, when aqueous phases are present both within and without the liposome, the lipid molecules will form a bilayer, known as a lamella, of the arrangement XY-YX.

Liposomes within the scope of the present invention can be prepared in accordance with known laboratory techniques. In one preferred embodiment, liposomes are prepared by mixing liposomal lipids, in a solvent in a container, *e.g.*, a glass, pear-shaped flask. The container should have a volume ten-times greater than the volume of the expected suspension of liposomes. Using a rotary evaporator, the solvent is removed at approximately 40°C under negative pressure. The solvent normally is removed within about 5 min. to 2 hours, depending on the desired volume of the liposomes. The composition can be dried further in a desiccator under vacuum. The dried lipids generally are discarded after about 1 week because of a tendency to deteriorate with time.

Dried lipids can be hydrated at approximately 25-50 mM phospholipid in sterile, pyrogen-free water by shaking until all the lipid film is resuspended. The aqueous liposomes can be then separated into aliquots, each placed in a vial, lyophilized and sealed under vacuum.

In the alternative, liposomes can be prepared in accordance with other known laboratory procedures: the method of Bangham *et al.* (1965), the contents of which are incorporated herein by reference; the method of Gregoriadis, as described in *DRUG CARRIERS IN BIOLOGY AND MEDICINE*, G. Gregoriadis, ed. (1979) pp. 287-341, the contents of which are incorporated herein by reference; the method of Deamer and Uster (1985), the contents of which are incorporated by reference; and the reverse-phase evaporation method as described by Szoka and Papahadjopoulos (1978). The aforementioned methods differ in their respective abilities to entrap aqueous material and their respective aqueous space-to-lipid ratios.

The dried lipids or lyophilized liposomes prepared as described above may be dehydrated and reconstituted in a solution of inhibitory peptide and diluted to an appropriate concentration with an suitable solvent, *e.g.*, DPBS. The mixture is then vigorously shaken in a vortex mixer. Unencapsulated adjuvant and antigen are removed by centrifugation at 29,000 × g and the liposomal pellets washed. The washed liposomes are resuspended at an appropriate total phospholipid concentration, *e.g.*, about 50-200 mM. The amount of antigen can be determined in accordance with standard methods. After determination of the amount of antigen encapsulated in the liposome preparation, the liposomes may be diluted to appropriate concentrations and stored at 4°C until use.

In a preferred embodiment, small unit vesicles, with or without phospholipid (phosphatidyl choline [PC] (*type III-B from bovine brain*)/ phosphatidyl serine [PS](*from bovine brain*), are prepared following the procedure described by Wilschut (1982) and Portoukalian *et al.* (1991). In brief, the sLe antigens, adjuvant and phospholipids are dissolved in equimolar ratio (*vide infra*) in chloroform:methanol (1:1, v/v) and evaporated to dryness in small round-bottom flasks over a rotary evaporator. The lipid layer formed in the bottom of the flask is then recovered as liposomes by adding 0.5 or 1 ml of warm sterile saline (40°C) and by intermittent vortexing and sonication for 30 min. The liposomes formed are not necessarily of uniform size but remain stable for more than one hour on ice. The liposomes may then be administered to elicit an immunotherapeutic response

ii. Porous Beads

Increasing density of expression of surface expression of antigens on artificial membrane is a major problem associated with induction of immune response. While both natural and artificial membranes are ideal, often other molecules such as neutral glycolipids and cholesterol on the membranes may interfere with immune response and may involve in immune recognition. To overcome this problem, porous bead made up of polystyrene and lactose may be used. The unique pore structure of the poly(styrene-divinylbenezene) (PSDVB) particles form the porous beads. These are inert matrices often used for antibody binding. These beads offers ideal material to attach the Sialyl Lewis antigens and one can monitor the density of incorporation. In addition these beads would provide the surface to accommodate other antigens related to sLe, such as gangliosides.

3. Immunotherapy

The results presented herein have significant relevance to immunotherapy of human diseases and disorders, including cancer. In human therapeutics it is important that the sLe antigen based therapeutic compositions of the present invention be formulated in a pharmacologically acceptable vehicle. Many such formulations are known, as described herein. In optimizing an immunotherapeutic for later use in humans, it is important that pre-clinical studies also be conducted in pharmacologically acceptable solutions, such as, by way of example only, RPMI 1640. Other suitable pharmacologically-acceptable media are described, *e.g.*, in Sigma Cell Culture, 1993 (incorporated herein by reference).

In using the immunotherapeutic compositions derived from the sLe/adjuvant incorporated cells of the present invention in treatment methods, other standard treatments may also be employed, such as radiotherapy or chemotherapy. However, it is preferred that the immunotherapy be used alone initially as it effectiveness can be readily assessed. Immunotherapies of cancer can broadly be classified as adoptive, passive and active specific, as described in the following sections.

It is contemplated that a wide variety of cancers may be treated using therapies described herein, such cancers include but are not limited to cancers of the brain (*e.g.*, glioblastoma, astrocytoma, oligodendroglioma, ependymomas), neurofibrosarcoma, meningia, lung, liver,

spleen, kidney, lymph node, pancreas, small intestine, blood cells, colon or colorectal region, stomach, thyroid, breast, endometrium, prostate, testes, ovary, skin, head and neck, esophagus, bone marrow, blood or other tissue.

The present invention is particularly suitable for use with other immunotherapeutic protocols. For example, pre-treatment with BCG is currently contemplated. Bast *et al.* (1974) reviewed the use of BCG in cancer treatment, giving substantial directions as to its various modes of uses. Minden *et al.* (1976) and Yamamoto *et al.* (1988) also reported on the mechanisms of action of BCG. Bennet *et al.* (1988) provided further evidence that pre-treatment with BCG is useful in the specific context of MPL.

A. Passive Immunotherapy

A number of different approaches for passive immunotherapy of cancer exist. They may be broadly categorized into the following: injection of antibodies alone; injection of antibodies coupled to toxins or chemotherapeutic agents; injection of antibodies coupled to radioactive isotopes; injection of anti-idiotype antibodies; and finally, purging of tumor cells in bone marrow.

In passive immunotherapy, patients are administered monospecific or polyspecific Mabs. In the case of melanoma, the antibodies used are often anti-ganglioside Mabs. Several anti-ganglioside antibodies (R24, 3F8 and Mab 36.1) have been tested in clinical trials (Vadhan-Raj *et al.*, 1988), but complete regression was rare. It is not clear that the antibody always reached its tumor target.

Preferably, human monoclonal antibodies, are employed in passive immunotherapy, as they produce few or no side effects in the patient. However, their application is somewhat limited by their scarcity and have so far only been administered intralesionally. Human monoclonal antibodies to ganglioside antigens have been administered intralesionally to patients suffering from cutaneous recurrent melanoma (Irie & Morton, 1986). Regression was observed in six out of ten patients, following, daily or weekly, intralesional injections. In another study, moderate success was achieved from intralesional injections of two human monoclonal antibodies (Irie *et al.*, 1989).

Certain limitations of passive immunotherapy are likely due to the interaction of administered antibodies with the tumor-derived antigens, again exemplified by the sLe antigens and gangliosides, shed into the blood. The present invention could be used prior to passive immunotherapy to achieve advantageous results. One would again administer to the animal or patient an immunologically effective amount of the adjuvant-incorporated cell composition, followed later by an immunologically effective amount of the polyclonal or monoclonal antibodies.

U.S. Patent 5,091,178 describes a method for treating human melanoma that includes administering a MG-21-like (HB-9011-like) Mab to a patient in a dose effective to kill melanoma cells. U.S. Patent 5,091,178 is incorporated herein by reference for the purpose of describing methods of passive immunotherapy that may be combined with the present invention. It is also used for the purpose of assisting in the identification effective therapeutic amounts of the adjuvant-incorporated cell compositions described herein. Thus, an amount of an adjuvant-incorporated cell composition that is effective to produce an amount of antibody equivalent to the amounts described in U.S. Patent 5,091,178 will likely be a therapeutically effective amount.

Systemic injection of unconjugated anti-ganglioside monoclonal antibodies was first carried out using an IgG_3 class murine monoclonal antibody, R24, in patients suffering from metastatic melanoma (Dippold *et al.*, 1980; 1984; 1985; Houghten *et al.*, 1985; Vadhan-Raj *et al.*, 1988). An effective treatment protocol was developed, which is also contemplated for use in passive immunotherapy in conjunction with the present invention. A phase 1 clinical trial of IL-2 and escalating doses of R24 has also been performed (Bajorin *et al.*, 1988).

The initial R24 treatment protocol includes systemic injection of ganglioside monoclonal antibodies at three dose levels (8, 80, or 240 mg/m^2) administered over a period of two weeks (Houghten *et al.*, 1985). Patients are given antibodies in 100 to 200 µl of 0.9% saline/5% human serum albumin. The effectiveness of the treatment protocol is dose dependent, however, so is the toxicity to the patient. Doses and treatment schedule may be varied in order to optimize immunotherapy.

It may be favorable to administer more than one monoclonal antibody directed against two different gangliosides or even antibodies with multiple ganglioside specificity. Treatment protocols may also include administration of lymphokines or other immune enhancers as in

Bajorin *et al.* (1988). The development of human monoclonal antibodies is described in further detail elsewhere in the specification.

It is contemplated that doses of about 24 million adjuvant-incorporated cells would be administered, containing about 25 ng to about 75 ng of MPL, with an average of about 50 ng of MPL.

B. Active Immunotherapy

In active immunotherapy, an autologous or allogeneic tumor cell composition or "vaccine" is administered, generally with a distinct bacterial adjuvant (Ravindranath & Morton, 1991; Morton & Ravindranath, 1996; Morton *et al.*, 1992; Mitchell *et al.*, 1990; Mitchell *et al.*, 1993). In melanoma immunotherapy, those patients who elicit high IgM response often survive better than those who elicit no or low IgM antibodies (Morton *et al.*, 1992). IgM antibodies are often transient antibodies and the exception to the rule appears to be anti-ganglioside or anticarbohydrate antibodies.

The use of adjuvant-incorporated tumor cell vaccines would be a valuable addition to active immunotherapy. Here, one could simply replace the irradiated tumor cells with an adjuvant-incorporated irradiated cell composition in accordance with the invention. Alternatively, one may still use the original irradiated tumor cell composition or "vaccine" and mix this with the same, or a related type, of irradiated tumor cells into which an adjuvant has been incorporated. Generally, the adjuvant-incorporated cells would be prepared first and then irradiated, although irradiation prior to or at any point of the preparative process is also possible.

Methods for treatment of melanoma with vaccines that include irradiated autologous melanoma tumor cells are well known, as exemplified by U.S. Patent 5,290,551, incorporated herein by reference. This patent concerns a haptenized tumor vaccine for the treatment of cancer where the cells are attached to dinitrophenyl, trinitrophenyl or N-iodoacetyl-N'-5 sulfonic 1-naphtyl ethylene diamine. Although the adjuvant-incorporated cell compositions of this invention are distinct from the compositions of the 5,290,551 patent, the method described in 5,290,551 could be employed in this invention.

An active specific immunotherapy protocol, developed by one of the present inventors (Morton *et al.*, 1992), involves immunization of melanoma patients with a polyvalent, irradiated melanoma cell vaccine (MCV). The patients are stratified by stage and disease status and given,

in a random manner, either pMCV alone or pMCV plus one of the biologic response modifiers (BRM), which have been shown to downregulate suppressor cell activity. These BRMs include Cimetidine (CIM; 1200 mg/d) (Smith/Kline, PA); Indomethacin (IND; 150 mg/d) (Lederle, NJ); or low-dose Cyclophosphamide (CYP; 75, 150 or 300 mg/m^2) (Johnson/Mead, NJ).

MCV is produced in large batches and analyzed for MAA antigen expression to determine variance between lots. The pMCV is screened for viral (HIV, hepatitis), bacterial and fungal infectious organisms. Equal amounts of each line are pooled to a total of 24 x 10^6 cells in serum-free medium containing 10% dimethyl sulfoxide and cryopreserved in liquid nitrogen. Before cryopreservation, the cells are irradiated to 100 GY.

Prior to treatment, pMCV is thawed and washed 3 times in phosphate buffered saline. pMCV is injected intradermally in axillary and inguinal regions on a schedule of every 2 weeks for six weeks, then monthly for a year. For the first two treatments, pMCV is mixed with BCG (Glaxo, England) (24 x 10^6 organisms/vial). After one year, the immunization interval is increased to every 3 months for one year, then every 6 months. Follow-up clinical and laboratory evaluations are repeated monthly, with chest x-rays every 3 months.

In using adjuvant-incorporated melanoma cell vaccines, as exemplified by MPL-MCV, one would again generally use about 24 million adjuvant-incorporated cells in the immunotherapy protocol described by Morton *et al.* (1992). These cells would contain upwards of about 25 ng of MPL, and generally between about 50 ng and about 75 ng of MPL. Although the amounts of MPL are not large, as the "parent" pMCV has already been shown to give beneficial results in patients, it is contemplated that the pMCV cells supplemented with virtually any amount of MPL in the membrane would give enhanced results. Where pre-treatment with BCG is used (Bast *et al.*, 1974; Bennet *et al.*, 1988) it is contemplated that a 3/4 reduced dose may be employed, *i.e.*, about 18 million cells.

Vosika *et al.* (1984) have shown that levels of MPL up to about 100 μg/m^2 (about 173 μg for the average adult) are safe for human administration. Therefore, the 50-75 ng of MPL currently proposed could also be increased considerably, either by using more cells or, preferably, by increasing the amount of MPL per cell, and still maintain the MPL dose clearly within the safety limits.

Using adjuvant-incorporated melanoma cell vaccines alone, as exemplified by MPL-MCV, one may be able to administer less cells than previously employed. This is based upon the following line of reasoning: tumors shed tumor-associated antigens into the general circulation; these antigens may be immunogenic or non-immunogenic, and may even be immunosuppressive; the shed immunosuppressive tumor antigens may cause immune exhaustion. One aspect of the present invention is to eliminate the immunosuppressive antigens and to restore immunocompetence. Coupled with the fact that the adjuvant-incorporated cells of the invention are highly effective due to the unique presentation of antigens and adjuvants together, this means that lower numbers of cells may prove to be effective in treatment methods.

C. Adoptive Immunotherapy

In adoptive immunotherapy, the patient's circulating lymphocytes, or tumor infiltrated lymphocytes, are isolated *in vitro*, activated by lymphokines such as IL-2 or transduced with genes for tumor necrosis, and readministered (Rosenberg *et al.*, 1988; 1989). This form of immunotherapy has produced several cases of regression of melanoma and renal carcinoma, but the percentage of responders were few compared to those who did not respond.

It is proposed that the failure of adoptive immunotherapy may be due to interaction of the activated cells with excess circulating tumor antigens, such as gangliosides, shed from tumor tissues. The present invention is ideally suited to overcome this problem. To achieve this, one would administer to an animal, or human patient, an immunologically effective amount of activated lymphocytes in combination with an adjuvant-incorporated cell composition as described herein. The activated lymphocytes will most preferably be the patient's own cells that were earlier isolated and from a blood or tumor sample and activated (or "expanded") *in vitro*.

In terms of the doses, it is contemplated that about 24 million adjuvant-incorporated cells would be injected intradermally, possibly followed by one to two further injections of 24 million adjuvant-incorporated cells. Currently, using MPL as an example, 24 million MPL-incorporated melanoma cells would contain, as a minimum, about 25 ng of MPL; on average, about 50 ng of MPL; and up to about 75 ng or so of MPL. This would be followed by the standard administration of IL-2 activated killer cells.

D. Biochemotherapy

Tumor cell resistance to DNA damaging agents represents a major problem in clinical oncology. One goal of current cancer research is to find ways to improve the efficacy of chemo- and radiotherapy. One way is by combining such traditional therapies with the immunotherapies outlined in the present invention.

To kill cells, inhibit cell growth, or metastasis, or angiogenesis, or otherwise reverse or reduce the malignant phenotype of tumor cells, using the methods and compositions of the present invention, one would generally contact a "target" cell with an immunotherapeutic composition based on the sLe antigenic compositions described herein and at least one other agent. These compositions would be provided in a combined amount effective to kill or inhibit proliferation of the cell. This process may involve contacting the cells with the immunotherapeutic agent and the agent(s) or factor(s) at the same time. This may be achieved by contacting the cell with a single composition or pharmacological formulation that includes both agents, or by contacting the cell with two distinct compositions or formulations, at the same time, wherein one composition includes the expression construct and the other includes the agent.

Alternatively, the immunotherapy treatment may precede or follow the other agent treatment by intervals ranging from minutes to weeks. In embodiments where the other agent and immunotherapy are applied separately to the cell, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the agent and immunotherapy would still be able to exert an advantageously combined effect on the cell. In such instances, it is contemplated that one would contact the cell with both modalities within about 12-24 hours of each other and, more preferably, within about 6-12 hours of each other, with a delay time of only about 12 hours being most preferred. In some situations, it may be desirable to extend the time period for treatment significantly, however, where several days (2, 3, 4, 5, 6 or 7) to several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

It also is conceivable that more than one administration of the immunotherapeutic composition of the present invention or the other agent will be desired. Various combinations may be employed, where immunotherapy is "A" and the other agent is "B", as exemplified below:

A/B/A B/A/B B/B/A A/A/B B/A/A A/B/B B/B/B/A B/B/A/B
A/A/B/B A/B/A/B A/B/B/A B/B/A/A B/A/B/A B/A/A/B B/B/B/A
A/A/A/B B/A/A/A A/B/A/A A/A/B/A A/B/B/B B/A/B/B B/B/A/B

Other combinations are contemplated. Again, to achieve cell killing, both agents are delivered to a cell in a combined amount effective to kill the cell.

Agents or factors suitable for use in a combined therapy are any chemical compound or treatment method that induces DNA damage when applied to a cell. Such agents and factors include radiation and waves that induce DNA damage such as, γ-irradiation, X-rays, UV-irradiation, microwaves, electronic emissions, and the like. A variety of chemical compounds, also described as "chemotherapeutic agents," function to induce DNA damage, all of which are intended to be of use in the combined treatment methods disclosed herein. Chemotherapeutic agents contemplated to be of use, include, *e.g.*, adriamycin, 5-fluorouracil (5FU), etoposide (VP-16), camptothecin, actinomycin-D, mitomycin C, cisplatin (CDDP) and even hydrogen peroxide. The invention also encompasses the use of a combination of one or more DNA damaging agents, whether radiation-based or actual compounds, such as the use of X-rays with cisplatin or the use of cisplatin with etoposide.

In treating cancer according to the invention, one may contact the tumor cells with an agent in addition to the immunotherapy. This may be achieved by irradiating the localized tumor site with radiation such as X-rays, UV-light, γ-rays or even microwaves. Alternatively, the tumor cells may be contacted with the agent by administering to the subject a therapeutically effective amount of a pharmaceutical composition comprising a compound such as, adriamycin, 5-fluorouracil, etoposide, camptothecin, actinomycin-D, mitomycin C, or more preferably, cisplatin. The agent may be prepared and used as a combined therapeutic composition, or kit, by combining it with the immunotherapeutic agent, as described above.

Agents that directly cross-link nucleic acids, specifically DNA, are envisaged to facilitate DNA damage leading to a synergistic, antineoplastic combination with the immunotherapeutic agents of the present invention. Agents such as cisplatin, and other DNA alkylating agents may be used. Cisplatin has been widely used to treat cancer, with efficacious doses used in clinical applications of 20 mg/m^2 for 5 days every three weeks for a total of three courses. Cisplatin is not absorbed orally and must therefore be delivered via injection intravenously, subcutaneously, intratumorally or intraperitoneally.

Agents that damage DNA also include compounds that interfere with DNA replication, mitosis and chromosomal segregation. Such chemotherapeutic compounds include adriamycin,

also known as doxorubicin, etoposide, verapamil, podophyllotoxin, and the like. Widely used in a clinical setting for the treatment of neoplasms, these compounds are administered through bolus injections intravenously at doses ranging from 25-75 mg/m^2 at 21 day intervals for adriamycin, to 35-50 mg/m^2 for etoposide intravenously or double the intravenous dose orally.

Agents that disrupt the synthesis and fidelity of nucleic acid precursors and subunits also lead to DNA damage. As such a number of nucleic acid precursors have been developed. Particularly useful are agents that have undergone extensive testing and are readily available. As such, agents such as 5-fluorouracil (5-FU), are preferentially used by neoplastic tissue, making this agent particularly useful for targeting to neoplastic cells. Although quite toxic, 5-FU, is applicable in a wide range of carriers, including topical, however intravenous administration with doses ranging from 3 to 15 mg/kg/day being commonly used.

Other factors that cause DNA damage and have been used extensively include what are commonly known as γ-rays, X-rays, and/or the directed delivery of radioisotopes to tumor cells. Other forms of DNA damaging factors are also contemplated such as microwaves and UV-irradiation. It is most likely that all of these factors effect a broad range of damage DNA, on the precursors of DNA, the replication and repair of DNA, and the assembly and maintenance of chromosomes. Dosage ranges for X-rays range from daily doses of 50 to 200 roentgens for prolonged periods of time (3 to 4 weeks), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely, and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells.

The skilled artisan is directed to "Remington's Pharmaceutical Sciences" 15th Edition, chapter 33, in particular pages 624-652. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

The inventors propose that the regional delivery of the immunotherapeutic agent to patients with cancers will be a very efficient method for delivering a therapeutically effective agent to counteract the clinical disease. Similarly, the chemo- or radiotherapy may be directed to a particular, affected region of the subjects body. Alternatively, systemic delivery of the

immunotherapeutic composition and/or the agent may be appropriate in certain circumstances, for example, where extensive metastasis has occurred.

In addition to combining immunotherapy with chemo- and radiotherapies, it also is contemplated that combination gene therapies will be advantageous. For example, targeting of p53 or p16 mutations at the same time may produce an improved anti-cancer treatment. Any other tumor-related gene conceivably can be targeted in this manner, for example, p21, Rb, APC, DCC, NF-1, NF-2, BCRA2, p16, FHIT, WT-1, MEN-I, MEN-II, BRCA1, VHL, FCC, MCC, *ras, myc, neu, raf, erb, src, fms, jun, trk, ret, gsp, hst, bcl* and *abl*.

4. Antibody Production

In addition to their uses as therapeutic agents in vaccines, the sLe antigens may be used for the large-scale production of antigen-specific T cells, and human and mouse polyclonal and monoclonal antibodies, for use in passive immunotherapy as discussed elsewhere in the specification. Means for preparing and characterizing antibodies are well known in the art (Antibodies: A Laboratory Manual, Cold Springs Harbor Laboratory, 1988).

Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogen comprising a polypeptide of the present invention and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically an animal used for production of anti-antisera is a non-human animal including rabbits, mice, rats, hamsters, pigs or horses. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

Antibodies, both polyclonal and monoclonal, specific an antigen may be prepared using conventional immunization techniques, as will be generally known to those of skill in the art. A composition containing antigenic epitopes of the compounds of the present invention can be used to immunize one or more experimental animals, such as a rabbit or mouse, which will then proceed to produce specific antibodies against the compounds of the present invention. Polyclonal antisera may be obtained, after allowing time for antibody generation, simply by bleeding the animal and preparing serum samples from the whole blood.

It is proposed that the monoclonal antibodies of the present invention will find useful application in standard immunochemical procedures, such as ELISA and Western blot methods

and in immunohistochemical procedures such as tissue staining, as well as in other procedures which may utilize antibodies specific to sLe antigens.

Means for preparing and characterizing antibodies are well known in the art (Antibodies: A Laboratory Manual, Cold Springs Harbor Laboratory, 1988). More specific examples of monoclonal antibody preparation are give in the examples below.

As is well known in the art, a given composition may vary in its immunogenicity. It is often necessary therefore to boost the host immune system, as may be achieved by coupling a peptide or polypeptide immunogen to a carrier. Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin can also be used as carriers. Means for conjugating a polypeptide to a carrier protein are well known in the art and include glutaraldehyde, *m*-maleimidobencoyl-N-hydroxysuccinimide ester, carbodiimide and bis-biazotized benzidine.

As also is well known in the art, the immunogenicity of a particular immunogen composition can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Exemplary and preferred adjuvants are discussed elsewhere in the specification

The amount of immunogen composition used in the production of polyclonal antibodies varies upon the nature of the immunogen as well as the animal used for immunization. A variety of routes can be used to administer the immunogen (subcutaneous, intramuscular, intradermal, intravenous and intraperitoneal). The production of polyclonal antibodies may be monitored by sampling blood of the immunized animal at various points following immunization. A second, booster, injection may also be given. The process of boosting and titering is repeated until a suitable titer is achieved. When a desired level of immunogenicity is obtained, the immunized animal can be bled and the serum isolated and stored, and/or the animal can be used to generate mAbs.

MAbs may be readily prepared through use of well-known techniques, such as those exemplified in U.S. Patent 4,196,265, incorporated herein by reference. Typically, this technique involves immunizing a suitable animal with a selected immunogen composition, *e.g.*, a purified or partially purified sLe antigen or cell expressing high levels of a sLe antigen. The immunizing

composition is administered in a manner effective to stimulate antibody producing cells. Rodents such as mice and rats are preferred animals, however, the use of rabbit, sheep frog cells is also possible. The use of rats may provide certain advantages (Goding, 1986), but mice are preferred, with the BALB/c mouse being most preferred as this is most routinely used and generally gives a higher percentage of stable fusions.

Following immunization, somatic cells with the potential for producing antibodies, specifically B-lymphocytes (B-cells), are selected for use in the mAb generating protocol. These cells may be obtained from biopsied spleens, tonsils or lymph nodes, or from a peripheral blood sample. Spleen cells and peripheral blood cells are preferred, the former because they are a rich source of antibody-producing cells that are in the dividing plasmablast stage, and the latter because peripheral blood is easily accessible. Often, a panel of animals will have been immunized and the spleen of animal with the highest antibody titer will be removed and the spleen lymphocytes obtained by homogenizing the spleen with a syringe. Typically, a spleen from an immunized mouse contains approximately 5×10^7 to 2×10^8 lymphocytes.

The antibody-producing B lymphocytes from the immunized animal are then fused with cells of an immortal myeloma cell, generally one of the same species as the animal that was immunized. Myeloma cell lines suited for use in hybridoma-producing fusion procedures preferably are non-antibody-producing, have high fusion efficiency, and enzyme deficiencies that render then incapable of growing in certain selective media which support the growth of only the desired fused cells (hybridomas).

Any one of a number of myeloma cells may be used, as are known to those of skill in the art (Goding, 1986; Campbell, 1984). For example, where the immunized animal is a mouse, one may use P3-X63/Ag8, P3-X63-Ag8.653, NS1/1.Ag 4 1, Sp210-Ag14, FO, NSO/U, MPC-11, MPC11-X45-GTG 1.7 and S194/5XX0 Bul; for rats, one may use R210.RCY3, Y3-Ag 1.2.3, IR983F and 4B210; and U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6 are all useful in connection with cell fusions.

In a preferred embodiment, human B cell lines that produce human monoclonal antibodies specific for sLe^x and sLe^a are established from B lymphocytes of patients vaccinated with sLe vaccine. The B cells are then immortalized using for example and EBV transformation technique. (Nagatsuka *et al.*, 1996). The transformed B-lymphoblastoid cells that produce

human monoclonal antibody to sLe antigens are then grown and the antibody produce is harvested, purified and stored for use in passive immunotherapy as described above.

Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually comprise mixing somatic cells with myeloma cells in a 2:1 ratio, though the ratio may vary from about 20:1 to about 1:1, respectively, in the presence of an agent or agents (chemical or electrical) that promote the fusion of cell membranes. Fusion methods using Sendai virus have been described (Kohler and Mil stein, 1975; 1976), and those using polyethylene glycol (PEG), such as 37% (v/v) PEG, by Gefter *et al.*, (1977). The use of electrically induced fusion methods is also appropriate (Goding, 1986).

Fusion procedures usually produce viable hybrids at low frequencies, around 1×10^{-6} to 1×10^{-8}. However, this does not pose a problem, as the viable, fused hybrids are differentiated from the parental, unfused cells (particularly the unfused myeloma cells that would normally continue to divide indefinitely) by culturing in a selective medium. The selective medium is generally one that contains an agent that blocks the *de novo* synthesis of nucleotides in the tissue culture media. Exemplary and preferred agents are aminopterin, methotrexate, and azaserine. Aminopterin and methotrexate block *de novo* synthesis of both purines and pyrimidines, whereas azaserine blocks only purine synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides (HAT medium). Where azaserine is used, the media is supplemented with hypoxanthine.

The preferred selection medium is HAT. Only cells capable of operating nucleotide salvage pathways are able to survive in HAT medium. The myeloma cells are defective in key enzymes of the salvage pathway, *e.g.*, hypoxanthine phosphoribosyl transferase (HPRT), and they cannot survive. The B-cells can operate this pathway, but they have a limited life span in culture and generally die within about two weeks. Therefore, the only cells that can survive in the selective media are those hybrids formed from myeloma and B-cells.

This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants (after about two to three weeks) for the desired reactivity. The assay should be sensitive, simple and rapid,

such as radioimmunoassays, enzyme immunoassays, cytotoxicity assays, plaque assays, dot immunobinding assays, and the like.

The selected hybridomas would then be serially diluted and cloned into individual antibody-producing cell lines, which clones can then be propagated indefinitely to provide mAbs. The cell lines may be exploited for mAb production in two basic ways. A sample of the hybridoma can be injected (often into the peritoneal cavity) into a histocompatible animal of the type that was used to provide the somatic and myeloma cells for the original fusion. The injected animal develops tumors secreting the specific monoclonal antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can then be tapped to provide mAbs in high concentration. The individual cell lines could also be cultured *in vitro*, where the mAbs are naturally secreted into the culture medium from which they can be readily obtained in high concentrations.

Monoclonal antibodies produced by either means may be further purified, if desired, using filtration, centrifugation and various chromatographic methods such as HPLC or affinity chromatography. Antibodies from serum can be purified using affinity perfusion chromatography as outlined in "Biomolecule Chromatography" (PerSeptive Biosystems, 1996).

5. Vaccination

The present invention contemplates vaccines for use in both active and passive immunization embodiments. Immunogenic compositions, proposed to be suitable for use as a vaccine, may be prepared most readily directly from immunogenic sLe antigens prepared in a manner disclosed herein. Preferably the antigenic material is extensively dialyzed to remove undesired small molecular weight molecules and/or lyophilized for more ready formulation into a desired vehicle.

The preparation of vaccines which contain sLe antigens as active ingredients is generally well understood in the art, as exemplified by U.S. Patents 4,608,251; 4,601,903; 4,599,231; 4,599,230; 4,596,792; and 4,578,770, all incorporated herein by reference. Typically, such vaccines are prepared as injectables. Either as liquid solutions or suspensions: solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified. The active immunogenic ingredient is often mixed with

excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants which enhance the effectiveness of the vaccines.

Vaccines may be conventionally administered parenterally, by injection, for example, either subcutaneously, intradermally or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkalene glycols or triglycerides: such suppositories may be formed from mixtures containing the active ingredient in the range of about 0.5% to about 10%, preferably about 1 to about 2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain about 10 to about 95% of active ingredient, preferably about 25 to about 70%.

The sLe antigens of the present invention may be formulated into the vaccine as neutral or salt forms. Pharmaceutically-acceptable salts, include the acid addition salts (formed with the free amino groups of the peptide) and those which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, including, *e.g.*, the capacity of the individual's immune system to synthesize antibodies, and the degree of protection desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner. However, suitable dosage ranges are of the order of several hundred micrograms active

ingredient per vaccination. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed by subsequent inoculations or other administrations.

The manner of application may be varied widely. Any of the conventional methods for administration of a vaccine are applicable. These are believed to include oral application on a solid physiologically acceptable base or in a physiologically acceptable dispersion, parenterally, by injection or the like. The dosage of the vaccine will depend on the route of administration and will vary according to the size of the host.

Various methods of achieving adjuvant effect for the vaccine includes use of agents such as aluminum hydroxide or phosphate (alum), commonly used as about 0.05 to about 0.1% solution in phosphate buffered saline, admixture with synthetic polymers of sugars (Carbopol®) used as an about 0.25% solution, aggregation of the protein in the vaccine by heat treatment with temperatures ranging between about 70° to about 101°C for a 30-second to 2-minute period, respectively. Aggregation by reactivating with pepsin treated (Fab) antibodies to albumin, mixture with bacterial cells such as *C. parvum* or endotoxins or lipopolysaccharide components of Gram-negative bacteria, emulsion in physiologically acceptable oil vehicles such as mannide mono-oleate (Aracel A) or emulsion with a 20% solution of a perfluorocarbon (Fluosol-DA®) used as a block substitute may also be employed.

In many instances, it will be desirable to have multiple administrations of the vaccine, usually not exceeding six vaccinations, more usually not exceeding four vaccinations and preferably one or more, usually at least about three vaccinations. The vaccinations will normally be at from two to twelve week intervals, more usually from three to five week intervals. Periodic boosters at intervals of 1-5 years, usually three years, will be desirable to maintain protective levels of the antibodies. The course of the immunization may be followed by assays for antibodies for the supernatant antigens. The assays may be performed by labeling with conventional labels, such as radionuclides, enzymes, fluorescents, and the like. These techniques are well known and may be found in a wide variety of patents, such as U.S. Patent Nos. 3,791,932; 4,174,384 and 3,949,064, as illustrative of these types of assays.

6. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1

Materials and Methods

Multivalent synthetic sialyl Lewis probes

The study of functional sialyl Lewis structures requires that the molecule be modified with reporter groups to allow detection and presentation in a biologically relevant multivalent format. A 30-kDa multivalent polymer is created by incorporating synthetic sialyl Lewis probes into a polyacrylamide matrix (GlycoTech, Rockville, MD). Approximately every fifth amide group of the polymer chain is N-substituted by the carbohydrate spacer arm. The polymers are also substituted with biotin in a 4:1 ratio. The multivalent biotinylated polymers are used with streptavidin reporter reagents for immobilization to microtiter plates in ELISA.

Monoclonal anticarbohydrate antibodies

The murine monoclonal antibodies (Mab) used in this study have been well characterized for their epitope specificity (Hanai *et al.*, 1986; Hanai *et al.*, 1990; Bara *et al.*, 1988; Kanako *et al.*, 1993; Hirabayashi *et al.*, 1986; Kuhn *et al.*, 1992). KM93 (antiSialyl Le^x IgM, affinity purified, 100 μg/500 μl) and KM 231(antiSialyl Le^a IgG2a, 100 μg/500μl) affinity purified) (Kamiya Biomedical, Seattle, WA) have been used as a cocktail for immunodiagnostic purposes (Hanai *et al.*, 1986; Hanai *et al.*, 1990). Monoclonal antibodies to Le^b (2.25LE, IgG1, affinity purified 200μg/200 μl) and Le^y (H18A, IgG3, affinity purified, 200μg/200 μl) (Seikagaku America, Ijamsville, MD) GD_3 (Mel-1, affinity purified IgG3, 225μg/ml, Signet Labs, Dedham, MA), MPL (8A1, affinity purified IgG1, 500 μg/100 μl, Centocor, Malvern, PA) KLH (KLH-60,

IgG2a culture supernatant, Sigma, St Louis, MO) were tested for their fine specificity before use. The commercial M2590 (ascites, Cosmo Bio Co., Ltd, Tokyo Kyoto-Ku, Tokyo 135) is not monospecific (Ravindranath *et al.*, 1996), but the only ganglioside expressed on cultured B16 melanoma cells is GM_3 (Yogeeswaran *et al.*, 1978). Unless specified otherwise, all monoclonal antibodies were diluted 1/100 with PBS (pH 7.2) containing 4% human serum albumin (HSA). Mouse IgG3, IgG1, IgG2a and IgM (Southern Biotechnology Associates Inc., Birmingham, Alabama) were used as negative controls that did not react with the antigens in ELISA plates. The control antibodies were only used after adjusting their protein concentration to that of their respective primary antibodies.

TABLE 2

List of Monoclonal antibodies used in this investigation

Antigen	Mab	isotype	purity (P)	concentration	Source	Ref.
Sialyl Le^x	KM93	IgM	affinity-P	100µg/500µl	Kamiya Biomed.	Hanoi *et al.*, 1986
Sialyl Le^a	KM231	IgG2a	affinity-P	100µg/500µl	Kamiya Biomed.	Hanoi *et al.*, 1990
GD_3	Mel-1	IgG3	affinity-P	225µg/ml	Signet Labs.	Pukel *et al.*, 1982
GD_2	14.G2a	IgG2a	cult. sup	-	Dr.R.Reisfeld	Reisfeld *et al.*, 1990

Source: Kamiya Biomedical, Seattle, WA.; Signet Laboratories, Dedham, MA.; Dr. Ralph Reisfeld Scripps Institute, La Jolla, CA.

Melanocytes and human and murine melanoma cells

Normal human epidermal melanocytes (NHEM 685; neonatal) were obtained from Clonetics (San Diego, CA) and were grown in Melanocyte Growth Medium (MGM) (500 ml) supplemented with MGM 3 Single-Quots (Clonetics, San Diego, CA). Human melanoma cells lines used in this study were the three cell lines constituting pMCV, namely, M10-v, M24 and M101. These cells were cryopreserved and thawed as described earlier (Morton *et al.*, 1992). Murine melanoma cells (B16) syngenic to C57BL/6J strain of mice were obtained from American Type Culture Collection (ATCC, Rockville, MD). All melanoma cell lines were cultured in RPMI-1640 medium (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal

bovine serum (FBS, Gemini Bioproducts, Calabassas, CA) and antibiotic-antimycotic (Pen-Strep-Fungizone, GibcoBRL 15240-013). The melanocytes, human melanoma cell lines, FBS and media were tested for mycoplasma (Mycotrim assay), HIV (PCR™) and HBV (PCR™) and found to be negative. Single cell suspensions of melanoma tumor cells were prepared from human tumor biopsies, after surgical resection following a procedure described elsewhere (Ravindranath *et al.*, 1996a). Briefly, minced tumor biopsies were incubated in 200,000 Kunitz of DNAse Type II (EC 3.1.21.1)/ 175,000 Kunitz of Collagenase Type I (EC 3.4.24.3) from *Clostridium histolyticum* in RPMI-1640 (1L) for 2 hr at 37°C. Single-cell suspensions were recovered for analysis after overlaying onto Ficoll-Paque (Pharmacia Biotech).

Mouse Immunization and challenge.

MCV was administered intradermally with or without adjuvants to 76 mice (C57BL/6J male, 6 wks old). The animals were divided into 8 groups as follows: sterile saline control (n=5), BCG alone (n=14), pMCV alone (n=9), pMCV-BCG (n=14), pMCV-MPL (n=13), pMCV-KLH (n=11), pMCV-MPL-BCG (n=6) and pMCV-KLH-BCG (n=4). For immunization, cells constituting vaccine were treated with 30 nmol MPL/10^6 cells and 75 nmol KLH/10^6 cells. The viability of pMCV, pMCV-BCG and pMCV-KLH was > 85% and that of pMCV-MPL and pMCV-MPL-BCG was 65 to 70%. The vaccine was administered intradermally on the posterior dorso-lateral regions of the body on weeks 0, 2, 4, and 6. On weeks -1, 0, 1, 3, 5, and 7, the animals were bled. Immunized animals were injected subcutaneously with 5×10^4 live B16-F1 melanoma cells. Tumor growth was measured in two dimensions with Vernier calipers and the values are expressed by multiplying the two dimensions. Selected mice that had become totally immobile because of tumor burden, had ceased feeding and drinking and were about to die were sacrificed for analyses of surface expression of the antigens. Five mice (one each from BCG, pMCV, pMCV-BCG, pMCV-MPL and pMCV-KLH) and six mice (including one from saline control) were sacrificed on day 33 and 40, respectively, and single-cell suspensions were prepared from the freshly resected tumors. Cells were cryopreserved as described earlier. The sacrificed mice were included for log rank survival analysis (by adding a day to their total days of survival). All animal studies were carried out at Harbor-UCLA Research and Education

Institute (REI), complying with regulations stipulated by the REI animal subjects committee (Project # 008239).

Immunization in guinea pigs

Thirteen guinea pigs (strain: HsD, Poc:DH, females, weight 300- 350 kg) were immunized on weeks 0, 2, 4 and 6. The vaccine formulations were administered intradermally in the right posterior dorso-lateral regions of the body. Each dose contained 1×10^7 cells consisting of an equal number of cells from each cell line. The animals were divided into four immunization groups: Group 1: pMCV with BCG (n = 4); Group 2, pMCV without BCG (n = 3); Group 3, pMCV-lysate with BCG (n = 3) and Group 4 pMCV-lysate without BCG (n = 3). BCG (1×10^6 bacterial cells) was admixed with pMCV (4×10^7) or pMCV-lysate (volume and the number of cells immunized as lysate are same as that used for pMCV) just before injection. Animals were bled on week -1, 0, 3, 5, 7 and 9.

Immunization in melanoma patients

Patients with American Joint Committee on Cancer (AJCC) stage III and IV melanoma were treated with pMCV consisting of three cryopreserved and irradiated allogeneic melanoma cell lines (M10-v, M24 and M101) as previously described (Morton *et al.*, 1992). pMCV has been well characterized and is produced under quality controlled manufacturing practice (Morton *et al.*, 1992). Patients were given pMCV on weeks 0,2,4, and every 4 wk thereafter for the first year. The first two treatments of pMCV (24×10^7 cells) were mixed with the Tice strain of *Bacillus Calmette Guerin* (24×10^8; prepared for human use)(Organon Teknika Co., Durham, NC). pMCV was administered intradermally at two sites in each axilla and groin at a total concentration of 24×10^7 cells consisting of an equal number of cells from each cell line. Patients entering the pMCV protocol met physical and disease status requirements as previously described (Morton *et al.*, 1992), and had received had no chemotherapy, radiotherapy or immunotherapy in the 30 days prior to commencing treatment. The patients underwent elective surgery to remove draining lymph nodes and/or other metastatic lesions within 15 wk of vaccine initiation. The pMCV treatment protocol was approved by the John Wayne Cancer Institute and Saint John's Hospital and Health Center human subjects protection committee and written informed consent of the patient was obtained.

Selection of adjuvants

The adjuvants used in this study include (1) a live culture preparation of BCG, an attenuated strain of *Mycobacterium bovis* (Tice strain; Organon Teknika Corporation, Durham, NC); (2) a de-acylated preparation of MPL from *Salmonella minnesota* R595 (Ribi, Hamilton, MT); and (3) keyhole limpet hemocyanin from *Megathura crenulata* (KLH, Boehringer-Mannheim, Indianapolis, IN). These 3 adjuvants exert profound effects upon the general immunological status of the recipient and can stimulate or depress specific responses against a variety of unrelated antigen materials presented in conjunction with these adjuvants. BCG has different immunological effects from MPL and KLH. The inventors have therefore included a combination of BCG with MPL or KLH. Some of the immunopotentiating properties of BCG include (i) regulation of lymphocyte trafficking in lymphoid organs; (ii) stimulation of proliferation and activation of splenic and thymic lymphocytes; (iii) augmentation of antibody production; (iv) enhancement of cells involved in antibody-dependent lysis of target cells; (v) activation of macrophages; and (vi) induction of lymphocytes to produce cytokines (Baldwin and Pimm, 1978). In addition, BCG-stimulated proliferating T lymphocytes may interact with macrophages that have ingested both BCG and cellular debris of tumor cells (Van der Meijden *et al.*, 1989). KLH has been found to selectively augment antibody response to tumor-associated ganglioside and carbohydrate antigens (Livingston, 1995). BCG and KLH are capable of inducing cellular and humoral response to itself, while MPL failed to induce antibody response to itself (Ravindranath *et al.*, 1994b). Some of the adjuvant roles of MPL include (i) induction of polyclonal-B-cell activation; (ii) inactivation of suppressor-T-cell activity in mice; (iii) stimulation of T cells and IFN-γ production; (iv) stimulation of T cells to enhance IL-1 secretion by macrophages; (v) activation of superoxide production, lysozyme activity, phagocytosis in macrophages; and (vi) augmentation of antibody production against T-cell-dependent and -independent antigens (such as gangliosides and carbohydrates) (Baker *et al.*, 1988; Baker *et al.*, 1994; Tomai and Johnson, 1989; Chen *et al.*, 1991; Elliott *et al.*, 1991; Myers *et al.*, 1995; Ravindranath *et al.*, 1994a; Ravindranath *et al.*, 1994b).

Incorporation of adjuvants onto pMCV cells.

Cultured M10-v, M24, and M101 melanoma cells were harvested with EDTA-dextrose buffer (Ravindranath *et al.*, 1996a) and washed with PBS-4% HSA. Viability was measured by trypan blue exclusion. Only cell populations with >85% viability were studied. Deacetylated MPL from *S. minnesota,* R595 (Ribi Immunochem Research, Hamilton, Montana) was incorporated onto cells in two steps: (Phillips *et al.*, 1990) An ethanolic suspension of MPL was mixed with RPMI-1640 (dilution 1/10) by sonication (3 min.) and vortexing in three cycles. (Walz *et al.*, 1990) An equal volume of cell suspension in RPMI was mixed with MPL in RPMI and incubated on a shaking water bath with intermittent vortexing or stirring with a magnetic stirrer at 37°C for 1 hr. Cells were then washed with PBS-4% HSA three times. Endotoxin-free, sterile KLH (Boehringer-Mannheim) was also adsorbed to cells by incubating the cells with the adjuvant suspended in RPMI at 37°C for 1 hr. After incorporating adjuvants, cell viability was monitored by Trypan-blue exclusion. Incorporation of the adjuvant into the glycocalyx was then assessed by Cs-ELISA or flow cytometry as described elsewhere (Ravindranath *et al.,* 1996, Ravindranath *et al.,* 1997a). The dilution of the anti-adjuvant antibodies was as follows: KLH-60 (1/100) and 8Al (1/32,000).

FACS analysis of incorporation of adjuvants.

MPL or KLH-treated cells (0.5×10^6) were suspended in 60 µl of RPMI-4% HSA in polypropylene tubes. 120µl of diluted first Ab (MAbs 8A1: 1/32,000, or MAb KLH-60, 1/100); and negative class-matched isotype controls (IgG1 or IgG2a adjusted to the protein concentration of the respective first antibody) was used. After gentle vortexing, the cells were incubated in a shaking water bath at 10°C for 90 min, then washed three times with cold PBS-4% HSA to remove unbound Ab. After the final wash, 100 µl of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse $F(Ab)_2$ IgG (Jackson ImmunoResearch, diluted 1/5000) was added to each vial and processed for FACS analysis as described elsewhere (Ravindranath *et al.*, 1996b).

Quantitation of cell surface antigens with cell suspension ELISA (Cs-ELISA)

The density of cell surface antigens and adjuvants was measured with cs-elisa (Ravindranath *et al.*, 1996) in cultured b16 murine melanoma cells, pMCV cells, and B16

melanoma cells from tumors of mice immunized with pMCV and pMCV-adjuvant formulations. Trypsin was not used due to its effects on cell surface antigen expression (Ravindranath *et al.*, 1996). A single-cell suspension (0.2 or 0.5 × 10^6 cells in 50 µl of solution) was used as either *background* (treated only with the second ab), *experimental* (treated with first and second ab) or *negative control* (treated with class matched isotypes of the first ab). 100 µl of the first antibody in pbs-4% hsa was added and incubated for 1.5 h at 10°C after washing the cells three times, 150 µl of a 1/5000 dilution of peroxidase-coupled goat anti-mouse ab was added and incubated for 1 h in a shaker at <10°C. After washing twice with pbs-hsa the cells were isolated and transferred to fresh vials containing 1 ml of pbs-4% hsa, and centrifuged to recover the pellet. Substrate solution (50 µl) was added to each vial, which was incubated in the dark at room temperature for 40 min., And the supernatant recovered after centrifugation. The substrate was transferred to microtiter wells containing 60 µl of 6N H_2SO_4. The absorbance was measured at 490 nm and 650 nm. The following controls were used: cells treated with substrate only (*blank*), cells treated with peroxidase-coupled second ab (*background*), and cells treated with non-specific purified isotypes corresponding to first ab (*negative control*). All values were adjusted to the background and corrected for negative control.

Characterization of the sLe ligands with Cs-ELISA

The carrier molecules of sLe structures can be proteins or lipids; consequently their immune responses may differ. Therefore, mild trypsinization was done by treating 0.5 × 10^6 cells (60 µl) with 0.01% trypsin (300 µl) at 37°C for 1 hr. The enzyme activity was arrested with RPMI-4% HSA. Cells were also treated with sialidase from *Vibrio cholerae* (Boehringer Mannheim) for 2 hrs at 37°C. To destroy the glycerol side chain of sialic acid, the free 1, 2 glycol of the glycerol side chain was cleaved by 5 mM sodium metaperiodate in PBS at 37°C for 1 hr. The antibody binding was assessed after washing viable cells (<80% total) 3x with RPMI-1640-4% HSA.

Anti-sLe Ab ELISA

Serum titers of anti-sLe antibodies were measured following an earlier protocol (Ravindranath *et al.*, 1994a). Microtiter plates (Falcon Probind 3915) were coated overnight with 100 µl of streptavidin (0.5 µg/well) (Sigma, St. Louis, MO) suspended in PBS (pH. 7.2) at

4°C. After washing excess streptavidin with PBS (pH 7.2), the plates were coated overnight with biotinylated-PAA-sLe polymer (3.3 pmole of sLe) (Glycotech, Rockville, MD) in carbonate buffer (pH 9.6). By box titration, the optimal concentration of the coated antigen was 3.3 pmol for sLe^x and for sLe^a. The serum Ab ELISA was carried out following a procedure described earlier (Ravindranath *et al.,* 1994c). After antigen coating and blocking with 4% HSA-PBS, the plates were incubated at 37°C for 90 min and exposed to sera or primary antibodies. Peroxidase-coupled secondary Ab, diluted 1/5000, was used o-PDE-H_20_2 was used as substrate as described earlier. Enzyme activity was arrested with sulfuric acid (60 µl of 6N), and the absorbance was measured at A 490 nm to A 650 nm. Controls were: wells without Ag, treated with second Ab and substrate (blank); wells without Ag, treated with first and second Ab and substrate (background); Ag-coated plates treated with class matched non-specific purified isotypes of primary Ab (negative control). All values were adjusted for non-specific binding. For purposes of comparison, pre- and post-vaccination sera of all groups were analyzed at the same time for a particular antigen.

Anti-sLe Ab ELISA after adsorption

Sera were also analyzed for anti-sLe IgM after adsorption against sLe^a or sLe^x. The biotinylated-antigens (3.3 pmole per 1.2 ml) were immobilized on streptavidin-coated 24-well plates. The sera of four patients were diluted 1/100, and 1.2 ml of diluted sera was overlaid and incubated at 37°C for 2 hr. Every two hours, the sera were transferred to new antigen-coated wells after retaining 400 µl for ELISA. The sera obtained after each adsorption were analyzed for anti-sLe^a and anti-sLe^x IgM titers. Control sera were treated likewise in wells without antigen. The percentage difference between experimental and control values was recorded.

ELISA to monitor serum sLe levels

An ELISA was developed to detect the levels of sLe antigens in fetal bovine serum (FBS). FBS (100 µl) was incubated with 0.01% Trypsin (400 µl) in PBS for 1 hr at 37°C. The mixture is made up to 1 mL by adding 500 µl of Tris buffer (pH. 9.5). Each well in a microtiter plate (Falcon Probind 3915) was coated with 20 µL of the trypsin digest of serum. To all wells, 180 µl of chloroform: ethanol (1:9) was added to facilitate uniform spreading of the tryptic digest and evaporated *in vaccuo* overnight. The non-specific sites were blocked with PBS-4% HSA,

pH 7.2 and incubated at 37°C for 60 min. Murine monoclonal sLe antibodies or the class matched isotypes (negative controls) were added at appropriate dilutions (1/1000). Subsequent procedure is same as described above for anti-sLe antibody ELISA (Ravindranath *et al.*, 1994c).

Measurement of delayed-type hypersensitivity (DTH) reaction:

The DTH reaction to the vaccine cells was studied one day after intradermal injection of 1×10^6 viable cells intradermally on weeks 0, 2, 4, and 6. Erythema and induration were measured with Vernier calipers in two dimensions. DTH expressed as the mean diameter of induration.

EXAMPLE 2

FIG. 1A shows that both sLe^x and sLe^a are co-expressed with other melanoma-associated gangliosides. One of the major melanoma-associated gangliosides is GD_3. Based on the relative expression of GD_3 and sLe^x, the inventors have identified two patterns in the cell surface expression of these antigens. (1) $GD_3 < sLe^x$: Tumor biopsies obtained from the alimentary system (liver, small bowl and intestine) show this pattern; (2) $GD_3 > sLe^x$: Tumor biopsies obtained from the subcutaneous metastasis show this pattern. The data not only suggest that the relative expression of the two antigens may vary with the site of metastasis but also vary depending on the routes of metastasis (hematogenous *vs* lymphatic). Presence of sLe^x and sLe^a in freshly obtained biopsies suggest that these antigens are of endogenous origin.

FIG. 1B shows the antigen expression in human melanoma cell lines grown in the medium (RPMI-1640) containing fetal calf serum (FCS). Based on the relative expression of GD_3 and sLe^x, the inventors have identified three patterns in the cell surface expression of these antigens. (1) $GD_3 < sLe^x$: This pattern is characteristic of human melanoma cell line M10-v; (2) $GD_3 > sLe^x$: This pattern is characteristic of human melanoma cell line M101; (3) high sLe^a and low sLe^x and GD_3: This pattern is characteristic of human melanoma cell line M24. The presence of sLe antigens in cultured cell lines could be due to exogenous origin, however analysis of sLe antigens in fetal calf serum revealed that sLe^x is highly negligible suggesting that they were not incorporated from the medium used for cell culture. Diversity in the profile of these antigens in the three cell lines represented above suggest that these cell lines are ideal candidates to be used as melanoma cell vaccine.

The antigen expression in polyvalent melanoma cell vaccine (pMCV) used in phase II and phase III clinical trial. pMCV constitutes the three human melanoma cell lines grown in the medium (RPMI-1640) containing fetal calf serum (FCS, FIG. 1C). When the combined pool of melanoma cell lines were analyzed, the inventors observed a difference in the relative expression of the antigens as follows: $sLe^a > sLe^x = GD_3 > GD_2$. When the three cell lines are combined, there was a decrease in the cell surface expression of sLe^x and GD_3, probably due to masking by other cell surface molecules.

The antigen expression in mouse B16 melanoma cell line grown in the medium (RPMI-1640) containing fetal calf serum (FCS) is shown in FIG. 1D. The B16 mouse melanoma cell line expresses a ganglioside GM_3. These cell lines do not express GD_3 or GD_2. However, B16 expresses sLe^x and sLe^a. Expression of sLe^x is significantly higher than sLe^a. Over expression of sLe^x in B16 cells and its absence in the culture medium used for the growth of the cells indicate that sLe^x in B16 is of endogenous origin. Expression of sLe^x in murine similar to human melanoma cell lines suggest that B16 would serve as an unique preclinical model for immunotherapy of melanoma.

The fine specificity and the epitope characteristics of the murine monoclonal antibodies used to identify sLe antigens are defined in FIG. 1E. The glycerol side chain of sialic acids is an important component of the epitopes recognized by these two antibodies. However these two Mabs are specific for their respective sLe structures suggesting that each Mab recognized NeuAcα2,3Gal residues and fucose differently based on the glycosidic linkage involving gal-GlcNAc and fucose-GlcNAc. Trypsin-treatment enables identification of the carrier molecule of sLe structures on the cell surface. Trypsin susceptibility reveals association with protein, and trypsin resistance indicates association with lipid moiety. In M10-v cells sLe^x is a component of the protein, whereas sLe^a in M24 cell line is a ganglioside. Vertical lines refer to standard deviation and the numbers above the bars refer to the number of analyses. Non-parametric two tailed t test was done to assess the level of significance, which is indicated in parentheses above the bars.

Passing M10-v cells in tissue culture and cryopreserving them does not affect the surface expression of sLe structures or the major gangliosides (FIG. 1F). This is also true for other cell lines. However, pMCV preparation is restricted to early passages. The vertical bars refer to standard deviation of four analyses. All analyses are carried out at the same time. Sensitivity,

reproducibility and reliability of cs-ELISA depend on the viability of the cells used in the assay The viability of cells used in this assay is indicated as a percentage.

The inventors document herein above the expression of sLe^x and sLe^a in human melanoma. The presence of the antigens in the cell surface of tumors derived from fresh biopsies show that the antigens are of endogenous origin. Since culture media and fetal calf serum (5%) used in the media did not contain this antigen in detectable levels, the inventors infer that the sLe may not be of exogenous origin. These antigens are not expressed in same proportion in all the cell lines. Based on the ratio of expression of sLe antigens and the major melanoma ganglioside,GD_3, the inventors categorized different subsets of cells, documenting the heterogeneity in the expression of the antigens in tumor cells (Table 3).

TABLE 3

Distinguishing immunochemical features of the glycocalyx of human melanoma cell lines used is a melanoma cell vaccine (MCV)

Melanoma cell lines	Immunochemical characteristics of the glycocalyx**
M10-v	$sLe^x > GD_3 > sLe^a = GD_2$
M24	$sLe^a >>> sLe^x > GD_3 > GD_2$
M101	$GD_3 > sLe^x \geq sLe^a > GD_2$

** Based on a double-blind analysis of three batches of each cell line.

Inventors clearly document that sLe^x is not expressed by normal melanocytes (Table 4), although previous workers have found the antigens in neutrophils activated by bacterial invasion. Although its biosynthetic precursors are blood group antigens (Lewis antigens) found on red blood cells, sLe antigens are not blood group antigens.

TABLE 4

Expression of sLe^a and sLe^x (expressed as absorbance) on the surface of normal human melanocytes *versus* cultured melanoma cells (positive control)

Human cells analyzed in Cs-ELISA s	Sialyl Lewis^a	Sialyl Lewis^x
Melanocytes (Source: Clonetics)	0.044 +/- 0.029 (5)	0 (5)
M24 Positive control for Sialyl Lewis^a	0.392 +/- 0.035 (5)	ND
M10-v Positive control for Sialyl Lewis^x	ND	0.371 +/- 0.024 (5)

0.2×10^6 cells were used with KM93 (IgM) for sLe^x and KM-231 (IgG1) for sLe^a. Values represent mean and standard deviation with sample size in parentheses. Values corrected against background and negative control. Class-matched isotypes were used to validate the results (negative control). ND: not tested.

In FIGs 1A through 1F the inventors also document the carrier molecules of sLe antigens. The inventors observe that sLe^x in a melanoma cell line, M10-v, is associated with the cell surface protein, whereas sLe^a is likely to be the moiety of a glycolipid on M24 cells. In addition the inventors demonstrate that expression of sLe antigens is persistent like other melanoma associated carbohydrate antigens and the expression is not affected by transferring cells from one passage to another. The inventors have observed the cell lines for fifty passages. Each passage of cells is grown for a week or two and then a fraction of cells were cryopreserved. After completing fifty passages, the cells were taken out of the freezer, thawed and analyzed for the cell surface expression. The remarkable consistency in the expression of sLe antigens on melanoma cell lines used as pMCV document the reproducibility of the cell vaccine preparation. Furthermore, the inventors attribute the success to achieve such consistent results to the sensitivity of the inventors novel cell-suspension ELISA (Ravindranath *et al*, 1996). The sensitivity of the assay depends not only on the monospecificity of the anti-sLe monoclonal antibodies, but also on the optimal viability (>85%) and density of cells required for the assay.

EXAMPLE 3

Sialyl Lewis^x and Sialyl Lewis^a are immunogenic in man:Clinical relevance

The immunogenicity of sLe^a and sLe^x in melanoma patients is documented by vaccinating melanoma patients with pMCV. All patients received pMCV (24 x 10^6 cells) and BCG (8 x 10^6 cells) for the first two immunizations (1° day 0 and 2° day 14) and pMCV alone in subsequent immunizations (3° day 28, 4° day 56, and 5° day 84). Day 0 refers to preimmune serum level of anti-sLe^a and anti-sLe^x IgM antibodies. Sera showed development of anti-sLe IgM but not IgG antibodies after immunization. The second immunizations clearly augmented the production of anti-sLe IgM antibody titers in three patients. The titer after third immunization was augmented in two patients, maintained in one, and declined in another. The profile of anti-sLe^a and anti-sLe^x IgM titers during the course of immunization remained identical in two patients and differed in the other two patients.

FIG. 2A shows the profile of a responder patient (# B0213) AJCC stage III melanoma. The development of anti-sLe^x IgM is observed after second immunization and continued until 3rd. The titer dropped three weeks after 3° immunization. In contrast, FIG. 2B depicts the profile of a non-responder patient (# B0725) AJCC stage III melanoma. In this case the sera showed unusually high titer before immunization and the titer declined after 1° and 2° immunizations. However 3° immunization caused an increase in the titers of both anti-sLe^x and anti-sLe^a IgMs.

In FIG. 2C the sera of responder patient (# B0815) showed continuous increase from first immunization until third immunization, after which the IgM levels for both anti-sLe^a and anti-sLe^x declined steadily. 3° and 4° immunizations did not induce antibody production.

The IgM antibody response to sLe^x and sLe^a differed in responder patient (# C0819). Anti-sLe^x IgM increased after first and second immunizations, and the level was maintained after 3° immunization.

Augmenting the antibody response against the sLe antigen may prevent tumor growth and metastasis. Inventors immunized melanoma patients with pMCV admixed with *Mycobacterium bovis* (BCG). Analysis of sera from 13 patients showed that 40% had high levels of anti-sLe^x IgM but low levels of anti-sLe^x IgG antibodies (Table 5). The inventors observe that sLe^x and sLe^a expressed on melanoma cells can be immunogenic. sLe^x and sLe^a

are associated with glycoprotein and glycolipid, respectively, and glycolipids are known to be poor immunogens (Yokoyama et al. 1963, Ravindranath et al, 1994). Therefore, the immune responses to sLe^x and sLe^a may differ depending on the carrier molecules (protein vs. lipid). Sera of normal volunteers and patients did not show a detectable level of IgG antibodies to sLe antigens. Anti-sLe IgM titers were low in normal and healthy individuals (Table 5), suggesting that these antibodies could be naturally occurring autoantibodies. The anti-sLe IgM titer in the sera from multiple volunteer normal donors was used as a baseline level of response to sLe^x and sLe^a (Table 5).

TABLE 5

Anti-sLe^x and anti-sLe^a IgM antibody titers in the sera of normal healthy volunteers susceptible to melanoma (Caucasian)

Parameters	Anti-sLe^a IgM	Anti-sLe^x IgM
n	18	20
Min.	69	54
Max.	302	627
Mean	140	267
SE(+/-)	17	40
Median	118	179
Mann-Whitney (two-tailed)		$p < 0.01$

In the preimmune sera of melanoma patients, the titer of IgM to sLe^x and sLe^a were increased at least threefold above the normal levels. Anti-sLe^a and anti-sLe^x IgM titers were measured four weeks after the first immunization. Patients were given pMCV on weeks 0,2,4, and every 4 wk thereafter for the first year. The first two immunizations were pMCV-BCG (on week 0 and 2) and subsequent immunizations were pMCV only. Several patients receiving pMCV developed at least a twofold increase in the titer of IgM against one or both of the sLe antigens (Table 6). Patients with a twofold or greater increase in IgM titer from the pre-immune level were considered responders. Six of 13 patients responded to sLe^x. Two of the six responders did not respond to sLe^a. The inventors have analyzed the post-immune IgM titers in

serial samples to determine whether the pMCV-induced anti-sLe antibody response is persistent or transient, as observed with antiganglioside antibody responses (Morton *et al*, 1992).

TABLE 6

Anti-sLe^x and anti-sLe^a IgM antibody titers in sera of melanoma patients before and after immunization with pMCV.

	Patient ID (St)*	Sialyl Lewis^x		Sialyl Lewis^a		Survival (mon)
		Preimmune	Postimmune**	Preimmune	Postimmune**	dsf/ovl)***
Responders	A1101 (IV)	200	1100	600@	308	62/62
	B0213 (III)	2000@	5800	322	1534	116/116
	B0815 (III)	560@	1380	596@	2778	96/96
	F0718 (III)	192	580	716	1289	46/76
	C0109 (III)	800@	>4000	800@	>4000	79/88
Non-	C0819 (III)	400	1100	309	1100	81/95
Responders	B0804 (III)	320	490	507	362	7/16
	B1023 (III)	670@	760	755@	513	47/70
	C1022 (III)	350	400	319	400	14/19
	C1021 (III)	180	250	260	377	2/14
	C0914 (III)	720@	917	726@	649	55/65
	G0809 (III)	327	382	154	280	3/9
	D0212 (III)	400	581	400	542	4/5

TABLE 6 - Continuted

Anti-sLe^x and anti-sLe^a IgM antibody titers in sera of melanoma patients before and after immunization with pMCV.

Patient ID (St)*	Sialyl Lewis^x		Sialyl Lewis^a		Survival (mon)
	Preimmune	Postimmune**	Preimmune	Postimmune**	dsf/ovl)***
Mean	548	1365	643	1164	
SE (+/-)	134	456	144	330	
n	13	13	13	13	
p (two-tailed *t*-test)		<0.05		<0.01	

(ST)* : American Joint Committee on Cancer Stage (AJCC stage);** Postimmune sera: antibody response against sLe antigens was observed within four to eight weeks after first immunization. The inventors' earlier observations on antiganglioside antibodies indicate that this time point is a reasonable reference to determine whether a patient has a specific antibody response to pMCV. Therefore, the inventors have assessed anti-sLe IgM titers before and after four to eight weeks of pMCV treatment. *** disease-free/overall survival (months); @ refers to patients with preexisting antibodies (high preimmune).

Analysis of the anti-sLe IgM titer in serial serum samples of four pMCV recipients is shown in FIGs 2A to 2D. The anti-sLe IgM titer reached a peak four to six weeks after treatment. First immunization with pMCV-BCG did not induce antibody response in most of the patients (CO819, BO815 and BO213), but the second immunization with pMCV-BCG induced a twofold or higher titer. One patient (B0725: non-responder) with high pre-immune anti-sLe titers showed a decline in anti-sLe^x and anti-sLe^a IgM titers during the first two immunizations but developed a threefold higher titer after the third immunization with pMCV alone.

TABLE 7

Serum anti-sLe^a and anti-sLe^x IgM reactivity after adsorption to sLe^a or sLe^x antigens*

Treatment of sera	Patient serum ID #	Percentage of IgM Adsorbed	
		sLe^a	sLe^x
	Unadsorbed	100%	100%
Adsorbed to sLe^a	C0819 (d.29)	31	0
	B0213 (d.50)	17	39
	B0815 (d.29)	50	38
	B0725 (d.0)	8	19
	Unadsorbed	100%	100%
Adsorbed to sLe^x	B0815 (d.28)	0	21
	B0213 (d.50)	0	0
	B0725 (d.0)	52	17
	C0819 (d.28)	29	24

* The values in Table 7 represent the average of the three adsorptions as described in the Material and Methods. Days refer to the days after immunization with pMCV. In order to distinguish whether anti-sLe^x and anti-sLe^a IgM are produced in response to different antigens or represent a cross-reacting species capable of binding to both sLe antigens, the inventors adsorbed the sera (1/100) with sLe^a or sLe^x. Table 7 shows IgM titers of the adsorbed sera measured using sLe^a or sLe^x in ELISA. The anti-sLe^a IgM level in the sera of patient C0819

adsorbed to sLe^a decreased by 31%, whereas anti-sLe^x IgM remained unaffected. Sera of patient B0815 adsorbed to sLe^x showed loss of anti-sLe^x IgM reactivity, whereas anti-sLe^a remained unaffected. However, other sera (B0213 [responder] and B0725 [non-responder]) showed loss of reactivity to both the antigens, suggesting the presence of cross-reactive IgM. The inventors observed that while both sLe^a and sLe^x elicited an antibody response and were immunogenic in melanoma patients, some patients were more responsive to one antigen than the other.

Overall and disease-free survival of patients correlated with high titers of anti-sLe^x antibodies. The inventors observe that patients with preexisting titers (>575) of antibodies survive better than others. Inventors also observe that patients with low preimmune titers (< 575) fall into two categories: Those who respond to vaccine by showing high titers of antibodies to sLe antigens, and those who do not respond to vaccine, the patients belonging to former category survive much better suggesting that vaccine containing sLe antigens are important for those patients who do not have high titers of antibodies. Inventors find that sLe antigen vaccine may help patients with high preimmune titer to maintain the high profile. The data described in the present invention for the first time, demonstrate the immunogenicity of sLe^x and sLe^a in humans. The observations clearly show that melanoma patients immunized with a cellular vaccine containing sLe^a and sLe^x induced a twofold or higher increase in IgM antibody titers. The overall and disease-free survival of the patients correlate with vaccine-induced antibody titers. These findings enable the inventors to infer that a sLe containing vaccine may be clinically beneficial for postoperative melanoma patients.

EXAMPLE 4

Does IgM antibody response induced by a vaccine containing sLe antigens possess antitumor activity: Testing in a preclinical model

Patients with preexisting or vaccine-induced anti-sLe IgM survive much better than non-responders. It is not clear whether anti-sLe IgM posses an antitumor activity. In order to assess whether anti-sLe IgM posses antitumor activity, the inventors have developed a preclinical murine model for melanoma. This preclinical model should meet the following criteria: (1) the tumor grown in animal model should express the target antigen (in this case, B16 mouse melanoma cells also express sLe^x and/or sLe^a); (2) the vaccine should include the target antigen

(in this case, sLe^x and/or sLe^a); (3) the vaccine should induce anti-sLe IgM antibodies; (4) Hypothetically, the immunized mice producing anti-sLe IgM should document anti-tumor activity. The antitumor activity can be measured in terms of (1) absence of metastatic spread of challenged tumor; (3) growth of tumor challenged after immunization and (4) survival of immunized mice after challenge of the tumor. The inventors observed that the transplanted tumor metastasized in mice that did not produce antibodies to this antigen. Mice immunized with pMCV-KLH produced very low level of antibodies and these mice developed lung metastasis, when challenged with syngenic B16 melanoma cells. This appears to be the first antitumor effect observed by the inventors. The inventors further analyzed both IgM and IgG antibodies and attempted to correlated the antibody titers with the tumor growth. Figures 3A, 3B and 3C document that mice immunized with different formulations of vaccine produced different species of antibodies. Mice immunized with pMCV only produce more of IgG antibodies to sLe^x and sLe^a. On the other hand, mice immunized with pMCV-BCG and pMCV-MPL produced equal or higher amount of IgM than IgG. Inventors observed that in the preclinical model, sLe-containing vaccine depending on the nature of the adjuvant added with the vaccine produced either IgG or IgM or both in varying ratios.

FIG. 3A shows that pMCV induces IgG and/or IgM antibody responses to sLe^x in the preclinical model depending on the adjuvant conjugated to pMCV. FIG. 3B shows that pMCV induces IgG and/or IgM antibody responses to sLe^a in the preclinical model depending on the adjuvant conjugated to pMCV. FIG. 3C. pMCV induces IgG and/or IgM antibody responses to the ganglioside GM_3 in the preclinical model depending on the adjuvant conjugated to pMCV. IgG and IgM antibody titers (values are represented as mean $\pm$SE in preimmune (n=16) and postimmune sera. Dotted line represents the preimmune level. Mice were intradermally immunized (a total of 4 immunizations on alternate weeks) with different formulations of vaccine.

The inventors document that in mice that produced high IgM to the sLe^x tumor growth is reduced. A series of figures 4A, and 4B, documented an association between high titers of IgM antibodies to sLe^x and reduced tumor growth. The same is true also for anti-GM_3 IgM. Although such a significant relationship was not observed with anti-sLe^a antibodies, anti-sLe^a

IgG:IgM ratio did show similar but not significant correlation. Based on these observation, the inventors inferred that anti-sLe IgM does indeed possess antitumor activity.

These experiments also lead to unanticipated results. Firstly, the inventors observed that the tumor growth is greater in mice that produced high IgG antibodies. Secondly the cells from these tumors failed to express the antigen. These features are illustrated in a series of figures (Figures 3,4 ,5 6 and 7).

FIG 4A, FIG. 4B, FIG. 4C and FIG. 4D show the nature and titer of antibody response to Sialyl Lewis antigens and the ganglioside GM_3 is correlated with tumor growth. After immunizing the mice with pMCV with different vaccine formulations, the mice were challenged with B16 melanoma cells and the growth of tumor was monitored. The antibody responses were correlated with the tumor size on day 35 after challenge. FIG. 4A depicts the correlation between mean anti-sLe^x IgG: IgM titer ratios and tumor growth in mice immunized with different vaccine formulations. The results of polynomial regression and r^2 are presented. r^2 indicates the frequency of the correlation. The slope is significant at $p < 0.05$. In FIG. 4B it can be seen that there is a significant linear relationship between anti-sLe^x IgM titer and tumor growth in mice immunized with different vaccine formulations. r^2 indicate that the frequency is 85%. FIG. 4C shows the correlation between mean anti-sLe^a IgG: IgM titer ratios and tumor growth in mice immunized with different vaccine formulations and FIG. 4D shows correlation between mean anti-GM_3 IgG: IgM titer ratios and tumor growth in mice immunized with different vaccine formulations.

The rate of tumor growth in mice varies depending on the vaccine-adjuvant combination. The differences in the antibody response to different vaccine-adjuvant combinations explain the mechanism underlying the regulation of tumor growth. Tumor growth in saline control represent the normal rate of growth. In mice immunized with pMCV or pMCV-MPL-BCG, tumor grew significantly better than in saline controls, whereas in mice immunized with pMCV-MPL or pMCV-BCG tumor growth was significantly slower than in saline controls. Other immunization groups were similar to the saline controls. The insert shows analysis of variance (ANOVA) on day 28. Sample size is same as indicated in the legend for figure 4. The sample size for saline control is 4.

Differences in overall survival between pMCV and saline control or pMCV-BCG or pMCV-MPL or BCG groups are indicated in FIG. 5B. The insert refers to test of equality over

strata, p values of Wilcoxan and Log-Rank tests and sample size of different experimental groups and control.

Tumor cells derived from challenge tumors grown in mice immunized with BCG, pMCV, pMCV-BCG, pMCV-MPL or pMCV-KLH were analyzed for expression of Sialyl Lewis antigens, GM_3 and cell-bound antibodies. FIG. 6A, FIG. 6B and FIG. 6C show cell-surface expression of sLe^a and sLe^x on tumors growing in immunized mice shows selective loss of expression of sLe^x. There is a selective loss of expression of Sialyl $Lewis^x$ in tumors grown in immunized mice. However, the expression of sLe^a was unaffected. Vertical bars refer to standard deviation of 4 analyses FIG. 6A. On the other hand, FIG. 6B shows that there was no loss of expression of GM3 in tumors grown in immunized mice. The tumor cells derived from challenge tumors grown in mice immunized with BCG, pMCV, pMCV-BCG, pMCV-MPL or pMCV-KLH. Expression of GM3 is unaffected in all the groups. There is not much difference in the expression of cell surface IgG and IgM among different immunization groups. However, when cell surface sLe antigens were measured, these values were used as background for correction (FIG. 6C).

FIG. 7 demonstrates the relationship between the ratio of IgG : IgM titers and tumor growth. This model proposes that IgG and IgM antibodies can be critical factors that affect the balance between tumor growth and host resistance. IgG antibodies, if mediate downregulation of antigen expression, may enable escape of tumor cells from immune attack. If titer of IgG is higher (which will be reflected in high ratio of IgG:IgM), greater will be the downregulation of antigen expression such as that of sLe^x . Lesser the ratio lesser the downregulation of the antigen. Cell surface expression of the antigen appears to be necessary prerequisite for IgM-mediated tumor killing as well as killing by cytotoxic T cells. The reduced growth correlates with high IgM, possibly due to IgM or T cell-mediated killing and incidentally lesser downregulation of the cell surface sLe^x of the challenged tumor. This model does not minimize the role of other antibodies directed to other antigens such as GM_3, which still can contribute to tumor regression independent of the mechanism proposed in this figure. This may explain why tumor growth is minimal in mice immunized with pMCV-MPL in spite of the ratio of anti-sLe^x IgG: IgM is close to one.

Thus, the two classes of antibody response to sLe^x distinguished favorable from unfavorable responses, and explain the apparent lack of survival benefit of immune responses seen in a number of clinical trials of vaccine therapy. Based on these observations, the inventors hypothesized that sLe^x may be a critical determinant of tumor metastasis and progression and that the antibody response to sLe^x may be correlated with clinical outcome in melanoma patients receiving vaccine therapy (Morton et al, 1992). Understanding the mechanisms of tumor suppression or enhancement after immunization with sLe^x may allow clinicians to develop passive or active specific immunotherapeutic strategies for treatment of melanoma and adjust therapeutic interventions according to a patient's immune response.

EXAMPLE 5

Survival of mice challenged with B16 tumor cells is related to type of adjuvant incorporated onto vaccine.

Eight treatment groups were evaluated for the effect of vaccines on overall survival. Of the 76 mice, 9 were immunized with pMCV, 12 with BCG or pMCV-BCG, 11 with pMCV-MPL and 9 with pMCV-KLH. All mice in the group immunized with pMCV perished by day 40 in contrast to 40% survival of saline controls. No mice receiving saline, pMCV, pMCV-KLH, pMCV-MPL-BCG or pMCV-BCG survived more than 60 days, whereas 20% of mice receiving BCG or pMCV-MPL survived. FIG. 5B illustrates the significant difference between overall survival of mice immunized with various adjuvant formulations of vaccine (MCV-BCG, pMCV-MPL, and BCG) compared to mice immunized with pMCV only.

EXAMPLE 6

Cellular cancer vaccine induces delayed-type of hypersensitivity reaction and augments antibody response to tumor-associated carbohydrate antigens (Sialyl Le^a, Sialyl Le^x, GD_3 and GM_2) better than soluble lysate cancer vaccine.

Sialyl Lewis antigens in the context of an intact membrane induces better antibody response in a guinea pig model. This is the fundamental basis for developing a vaccine in the context of a membrane. Allogenic whole cell and lysate cancer vaccines are associated with very different clinical outcome, which could be due to different immune responses to critical tumor-

associated antigens. In this Example, a guinea pig model was used to evaluate the immune responses to melanoma-associated carbohydrate antigens administered in whole cell and soluble lysate vaccines produced from the same cell lines and administered with or without Bacilli Calmette-Guerin (BCG).

Glycocalyx of pMCV: The three human melanoma cell lines constituting pMCV expressed the gangliosides GD_3, GD_2, and oligosaccharide residues of sLe^a and sLe^x (FIG. 1B). M24 cells overexpressed sLe^a and expressed small amounts of sLe^x. M10-v cells expressed a large amount of sLe^x, whereas M101 cells expressed equal amounts of sLe^x and sLe^a. The profile presented in FIG. 1C shows that the predominant constituent of the glycocalyx of pMCV is sLe^a, with GD_3 and sLe^x expressed in equal amounts.

DTH reaction to pMCV: No animal developed a DTH response to pMCV prior to immunization. A mild (9/13 animals) to prominent (4/13) erythema with or without induration was seen in most animals after the first immunization. FIG. 8A shows that pMCV with BCG induced a significantly higher (p=0.05) DTH response than pMCV-lysate with BCG. No DTH response was observed before immunization. No distinct induration was seen after the first immunization. The size of induration increased after the third immunization. 1×10^6 viable, irradiated pMCV cells were injected intradermally in guinea pigs. The viability of pMCV was > 85%. (all) refers to vaccine with or without BCG. The vertical bars represent standard deviation. The sample size for each experiment is indicated above the vertical bars. Non-parametric t-test values are indicated.

Thus FIG. 8A shows that there is a delayed-type hypersensitivity reaction to xenogeneic pMCV in the skin of guinea pigs after the fourth immunization with different formulations of pMCV.

Augmentation of antibodies to melanoma-associated sLe^x, sLe^a, GD_3, GD_2 and GM_2 : To test the hypothesis that tumor cell viability and membrane integrity is required for immune recognition and antibody response for major carbohydrate antigens, the inventors compared the IgG antibody response induced by pMCV and pMCV-lysate. The IgG antibody response after four immunizations was highest for GD_3, followed by GD_2, GM_2, and then sLe antigens (Table 8).

TABLE 8

Preimmune and postimmune serum IgG titers in guinea pigs

Antigen (per well)	Serum IgG titers after fourth immunization : Number of fold increase from preimmune titer/ Preimmune titer (n = 10)	MCV + BCG (n = 4)	MCV (n = 3)	MCV-lysate + BCG (n = 3)	Lysate (n = 3)
sLe^a (3.3 pmol)	164 ± 22	x3	x1	x<2	x1
sLe^x (3.3 pmol)	189 ± 41	x3	x2	<2	x1
GD_3 (1 pmol)	190 ± 94	x7	x6	x6	x3
GD_2 (1 pmol)	90 ± 45	x5	x3	x3	x3
GM_2 (1 pmol)	71 ± 30	x4	x2	x2	-

Anti-sLe^a IgG antibody response:

FIG. 8B and FIG. 8D shows that the titer of anti-sLe^a IgG differed significantly between prevaccine and postvaccine sera in animals immunized with pMCV-BCG. The increase in antibody titer is significantly higher ($p < 0.001$) after the fourth immunization after Bonferoni adjustment for sample size. A similar significant ($p < 0.01$) increase was observed in animals immunized with pMCV without BCG. However, no such increase was observed in animals immunized with pMCV-lysate-BCG or pMCV-lysate only.

Anti-SLe^x IgG antibody response:

FIG. 8C and FIG 8D shows that the titer of anti-sLe^x IgG is significantly higher after four immunizations with pMCV-BCG. The serum anti-sLe^x IgG titers did not vary between pre- and postimmunization in animals immunized with pMCV without BCG or with pMCV-lysate vaccine with or without BCG.

These data demonstrate that both sLe^a and sLe^x are immunogenic in guinea pigs. After four immunizations, pMCV-BCG induced significantly high antibody response to sLe^x (FIG. 8B) and sLe^a (FIG. 8C). The major gangliosides of pMCV are GM_2, GD_3 and GD_2 the serum IgG response to these gangliosides was also monitored by the inventors. The serum anti-GM_2 IgG

antibodies showed a significant increase after the fourth immunization with pMCV-BCG. No such increase was observed after immunization with pMCV without BCG or with pMCV-lysate vaccines with or without BCG. Serum anti-GD_3 IgG also increased significantly after four immunizations with pMCV-BCG ($p < 0.005$), pMCV-lysate-BCG ($p< 0.02$) and pMCV without BCG ($p < 0.05$). The anti-GD_3 IgG antibody response also differed significantly between pMCV with and without BCG ($p=0.05$). Results obtained with anti-GD_2 IgG titers are strikingly different from those observed with other antigens. All vaccine formulations induced a significantly ($p < 0.05$) higher response to this antigen and there was no difference among the different vaccine groups after four immunizations. Thus pMCV-lysate with or without BCG was poorly immunogenic.

FIG. 8D summarizes the superiority of whole cell vaccine in inducing antibody response to sLe^a and sLe^x. The preimmune value is the mean of eight analyses. The results of analyses of variance as well as the levels of significance among pre- and post-immune groups are provided after Bonferoni adjustment for the sample size. The vertical bars in immunization groups refer to standard deviation of 4 analyses.

These data show that sLe antigens induce immune response in the context of an integrated membrane. To document this, the inventors have immunized cellular vaccine expressing sLe antigens with or without an adjuvant and soluble lysate vaccine expressing the same amount of sLe antigens with or without an adjuvant. The results presented in the FIG. 8B-8D document the statistical analysis of the differences in the vaccine induced immune response. Clearly sLe antigens expressed in the context of a biological membrane elicits better antibody response than the antigens in soluble lysate form.

This study indicates that whole cell vaccine is superior to soluble or lysate vaccine because it induces a better immune response against cell-surface antigens. The addition of BCG significantly increased the antibody response suggesting that an exogenous adjuvant may immunopotentiate antigens better in the presence of an intact cell membrane.

EXAMPLE 7

Use Of Pmcv Expressing Sialyl Lewis Antigens, To Augment Immune Response In Colorectal Cancer Patients Expressing The Antigens In The Autologous Tumors.

There are approximately 160, 000 new cases of colon cancer every year, leading to over 60, 000 deaths. At present, there appears to be no effective therapeutic intervention available. The aim of the studies presented in this Example was to determine whether pMCV expressing sialyl Lewis antigens, the antigens shared by colorectal carcinomas, augments immune response in colorectal cancer patients expressing the antigens in the autologous tumors.

Patients were immunized with 24 million cells of cancer vaccine (irradiated and cryopreserved) on weeks 0, 2, and 4 and every 4 weeks thereafter. The first two immunizations consisted of vaccine cells mixed with the BCG (Tice) (8 million). Patents were bled on day 0 and on days of immunization. Studies were carried out on ten colon cancer patients who have received vaccine. These patients were designated SD80X, WB68X, VG75X, AS64X, SS68X, ES50+AWD; CO73 +NED; CF70+AWD; PC46X and MM46+NED

Serial bleeds of each patient (a total of 94 sera collected in five years) were analyzed for antibodies to nine gangliosides and sialyl Lewis antigens. The values are expression in titers.

The results of these studies are shown in FIG. 9A (anti-sLe^X IgM) and FIG. 9B (anti-sLe^A IgM). From these figures it is clear that individuals with colon cancer had high levels of IgM antibodies to sialyl Lewis (sLe) antigens before immunization. It was also found that 80% of colon cancer patients responding to ganglioside GM2 had high titers of preexisting IgM against sLe antigens. Furthermore, pMCV expressing sialyl Lewis antigens prolonged the titers of preexisting IgM and augmented the titers of anti-sLe^X IgM in two of ten patients (SD80X; SS68X). These two patients were at stage IV disease at the time of immunization and survived 43 and 34 months, respectively, since initiation of vaccine. pMCV also augmented the titers of anti-sLe^A IgM in two of ten patients (SD80X; CO73+NED).

The inventors conclude that sLe antigenic compositions are immunogenic in colorectal cancer. Further, patients that have preexisting or vaccine induced high titers against these antigens show prolonged survival.

* * *

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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U.S. Patent No. 4,579,945

U.S. Patent No. 4,866,034

U.S. Patent No. 4,877,611

U.S. Patent No. 4,950,645

U.S. Patent No. 4,987,237

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CLAIMS:

1. An antigenic composition comprising a Sialyl Lewis antigen and an adjuvant.

2. The composition of claim 1, wherein said Sialyl Lewis antigen is Sialyl Lewis^x or Sialyl Lewis^a.

3. The composition of claim 2, wherein said Sialyl Lewis antigen is Sialyl Lewis^x.

4. The composition of claim 1, wherein said composition comprises a plurality of Sialyl Lewis antigen species.

5. The composition of claim 1, wherein said Sialyl Lewis antigen is contained in the carbohydrate moiety of a glycoprotein, mucin or glycolipid.

6. The composition of claim 5, wherein said glycoprotein is CEA or MUC-1.

7. The composition of claim 4, wherein said composition comprises Sialyl Lewis^x and Sialyl Lewis^a.

8. The composition of claim 4, further comprising one or more tumor-associated ganglioside antigen species.

9. The composition of claim 1, wherein said Sialyl Lewis antigen is a multimer.

10. The composition of claim 9, wherein said multimer is a dimer, a trimer, a tetramer, a pentamer, a hexamer, a septamer, an octamer, a nonamer or a decamer.

11. The composition of claim 1, wherein said Sialyl Lewis antigen is a heteromer.

12. The composition of claim 1, wherein said Sialyl Lewis antigen is in a soluble form.

13. The composition of claim 12, wherein said Sialyl Lewis antigen is conjugated to said adjuvant.

14. The composition of claim 1, wherein said adjuvant is a biopolymer or a biomembrane.

15. The composition of claim 14, wherein said biomembrane is a bacterial membrane.

16. The composition of claim 15, wherein said bacterial membrane is derived from a bacterial genus from the group consisting of *Mycobacterium, Salmonella, Escherichia, Heliobacter, Staphylococcus* and *Streptococcus*.

17. The composition of claim 16, wherein said composition comprises a bacterial coat, a bacterial polysaccharide, a bacterial glycolipid, bacterial nucleic acid, bacterial lipid or bacterial protein.

18. The composition of claim 1, wherein said composition further comprises at least a first cell, wherein said Sialyl Lewis antigen is disposed on the cell membrane of said cell.

19. The composition of claim 18, wherein said cell is a human cell.

20. The composition of claim 18, wherein said cell is a murine cell.

21. The composition of claim 18, wherein said cell is a guinea pig cell.

22. The composition of claim 18, wherein said cell is a fibroblast.

23. The composition of claim 19, wherein said cell is a tumor cell.

24. The composition of claim 23, wherein said tumor cell is irradiated.

25. The composition of claim 19, wherein said cell is an erythrocyte.

26. The composition of claim 23, wherein said cell is a melanoma cell.

27. The composition of claim 26, wherein said melanoma cell is irradiated.

28. The composition of claim 27, wherein said melanoma cell is M27, M18, M14, M111, M22, M7, M102, M108, M16, M104, M109, M25, M24, M10 or M101.

29. The composition of claim 28, wherein said melanoma cell is M24, M10 or M101.

30. The composition of claim 18, further comprising a tumor-associated ganglioside.

31. The composition of claim 30, wherein said ganglioside is a cell surface ganglioside.

32. The composition of claim 30, wherein said ganglioside is a neural or extraneural tissue ganglioside.

33. The composition of claim 30, wherein said ganglioside is selected from the group consisting of GD_3, GD_2 or GM_2, GM_3, GM_{1a}, GM_{1b}, GD_{1a}, GD_{1b}, GT_{1a}, GT_{1b}, GT_3 and GQ_{1b}.

34. The composition of claim 18, further comprising at least a second cell that is genetically distinct from said first cell.

35. The composition of claim 1, wherein said adjuvant is incomplete Freund's, complete Freund's, bacterial cell wall, KLH, LTA, GTA, Chitin, MDP, threonyl-MDP, MTPPE, BCG, cell wall skeleton, trehalose dimycolate, QS21, Quil A or lentinen.

36. The composition of claim 35, wherein said adjuvant is BCG.

37. The composition of claim 36, comprising a population of cells that includes between about 3 to about 20 x 10^6 BCG organisms and about 24 x 10^6 cells.

38. The composition of claim 1, wherein said adjuvant is a lipopolysaccharide group adjuvant.

39. The composition of claim 1, wherein said adjuvant is a detoxified endotoxin.

40. The composition of claim 1, wherein said adjuvant is lipopolysaccharide, lipid A, monophosphoryl lipid A (MPL) or derivatives thereof.

41. The composition of claim 37, comprising a population of cells that includes between about 24 x 10^6 and about 200μg of MPL per 10^6 cells.

42. The composition of claim 1, dispersed in a pharmacologically acceptable formulation.

43. The composition of claim 18, wherein at least a second Sialyl Lewis antigen species is disposed on the cell membrane of said cell.

44. The composition of claim 14, further comprising a liposomal biomembrane.

45. The composition of claim 44, wherein said liposomal biomembrane is one or more of a unilamellar, multilamellar or cochlear vesicle.

46. The composition of claim 14, further comprising a biopolymer made of porous polystyrene [poly(styrene-divinylbenzene)] (PSDVB).

47. The composition of claim 18, wherein said cell is genetically engineered to express an exogenous gene.

48. The composition of claim 47, wherein said exogenous gene encodes a cytokine, a Sialyl Lewis antigen, an adjuvant, a glycosylating enzyme or a Sialyl Lewis antigen carrier.

49. The composition of claim 18, wherein said cell is genetically engineered to overexpress a normal cell product.

50. The composition of claim 49, wherein said normal cell product is selected from the group consisting of a Sialyl Lewis antigen or a cytokine.

51. The composition of claim 35, wherein said Sialyl Lewis antigen is covalently conjugated to said adjuvant.

52. A vaccine comprising a Sialyl Lewis antigen and an adjuvant in a pharmacologically acceptable buffer, diluent or excipient.

53. A method of stimulating an immune response in an animal comprising:

a) providing a pharmacologically acceptable composition comprising a Sialyl Lewis antigen and an adjuvant; and

b) administering said composition to said animal in an amount effective to stimulate an immune response against said Sialyl Lewis antigen.

54. The method of claim 53, wherein said Sialyl Lewis antigen is disposed on the surface of a cell.

55. The method of claim 54, wherein said cell is a tumor cell.

56. The method of claim 55, wherein said cell is an irradiated tumor cell.

57. The method of claim 56, wherein said cell is an autologous tumor cell obtained from said animal.

58. The method of claim 56, wherein said cell is an allogenic tumor cell.

59. The method of claim 56, wherein said cell is an erythrocyte obtained from said animal.

60. The method of claim 53, wherein said composition is administered to said animal by injection.

61. The method of claim 53, wherein said animal has a solid tumor and said composition is administered to said animal by injection into the tumor site.

62. A method for treating a tumor in an animal comprising:

a) providing a composition comprising a Sialyl Lewis antigen and an adjuvant; and
b) administering said composition to said tumor in an amount effective to inhibit the growth of said tumor.

63. A method for inducing a predominantly IgM response in an animal comprising:

a) providing a pharmacologically acceptable composition comprising a Sialyl Lewis antigen and an adjuvant; and
b) administering said composition to said animal in an amount effective to stimulate an IgM response.

64. A method for screening a composition for the ability to induce an anti-tumor immune response in an animal comprising:

a) providing a pharmacologically acceptable composition comprising a Sialyl Lewis antigen and an adjuvant;

b) administering said composition to said animal in an amount effective to stimulate an immune response; and

c) determining the extent of an IgM response in said animal against said Sialyl Lewis antigen.

65. A kit comprising, in suitable container means, a pharmaceutically acceptable composition comprising a Sialyl Lewis antigen and an adjuvant.

66. A human monoclonal antibody that reacts immunologically with a Sialyl Lewis antigen, wherein said monoclonal antibody is of the IgM class.

67. A method of isolating a Sialyl Lewis specific B cell or plasma cell comprising:

a) administering the vaccine of claim 50 to a subject; and

b) isolating said B cell or plasma cell.

68. The method of claim 67, further comprising immortalizing said B cell.

69. The method of claim 68, further comprising:

a) culturing said B cell; and

b) purifying antibodies generated by said B cell.

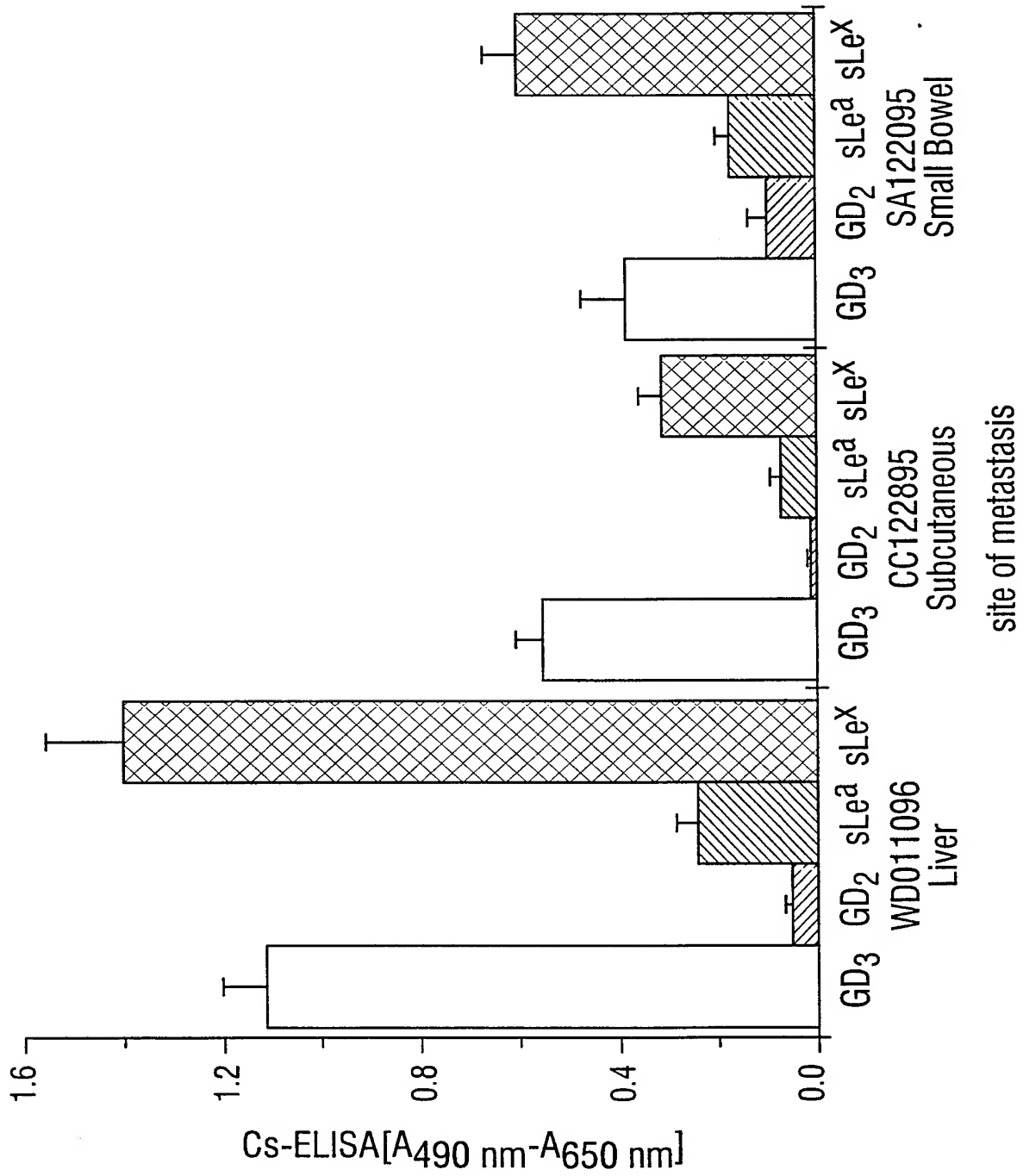
Cs-ELISA[A490 nm-A650 nm]
1.6
1.2
0.8
0.4
0.0
GD3
GD2
sLea
sLeX
WD011096
Liver
CC122895
Subcutaneous
site of metastasis
SA122095
Small Bowel

FIG. 1A

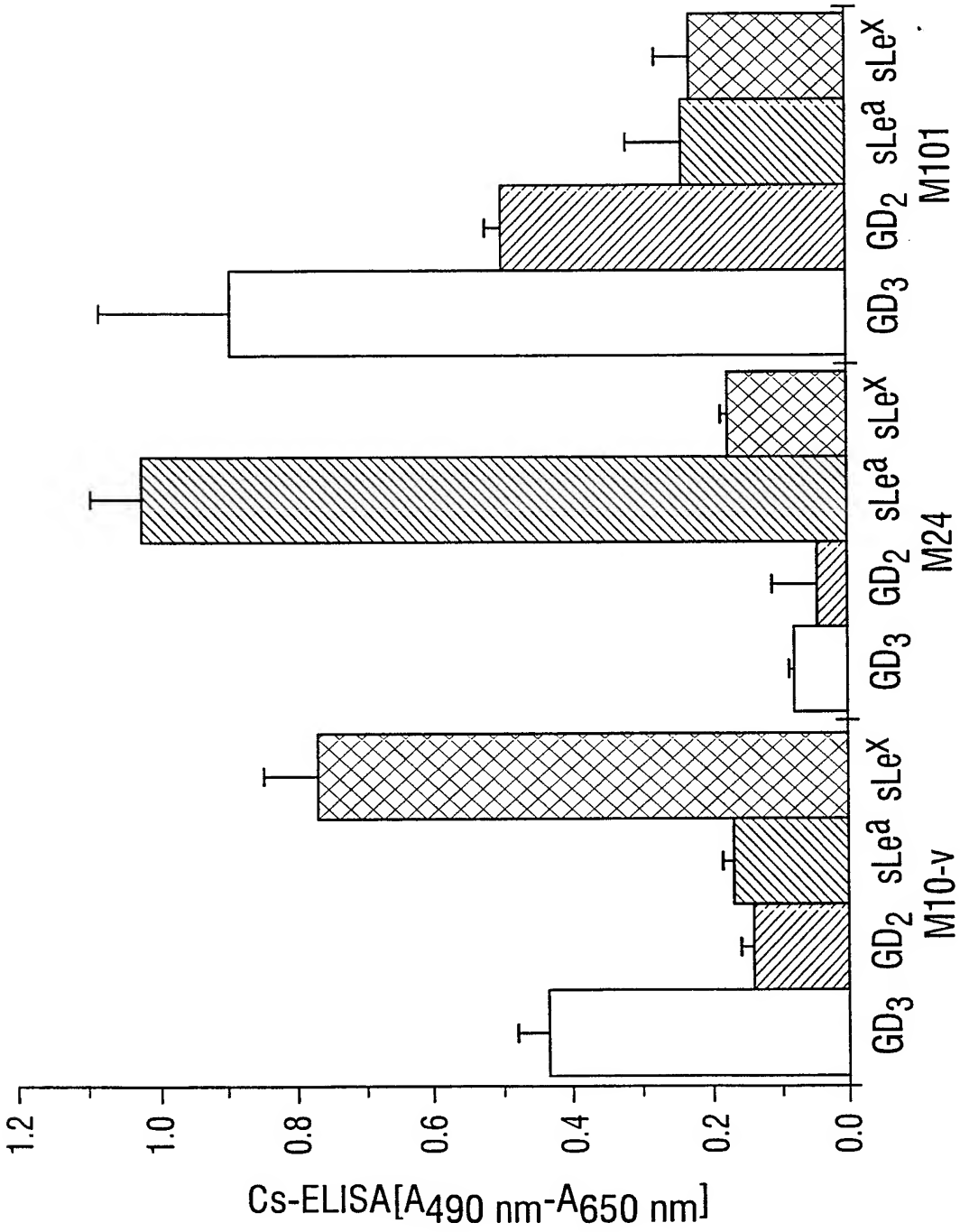
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Cs-ELISA[A490 nm-A650 nm]
GD3
GD2
sLea
sLex
M10-v
M24
M101

FIG. 1B

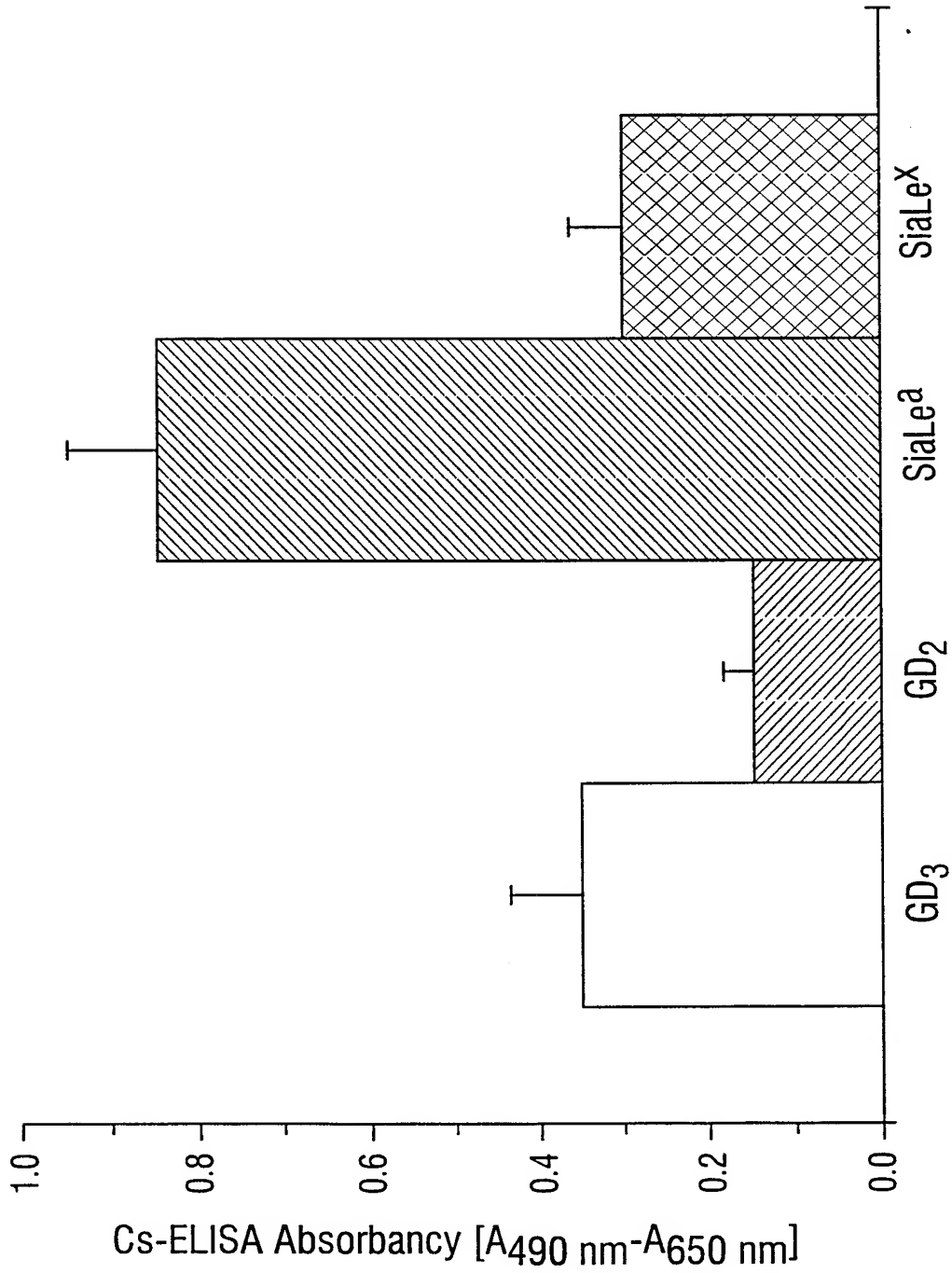
SiaLe^X
SiaLe^a
GD_2
GD_3
1.0
0.8
0.6
0.4
0.2
0.0
Cs-ELISA Absorbancy [$A_{490\ nm}$-$A_{650\ nm}$]

FIG. 1C

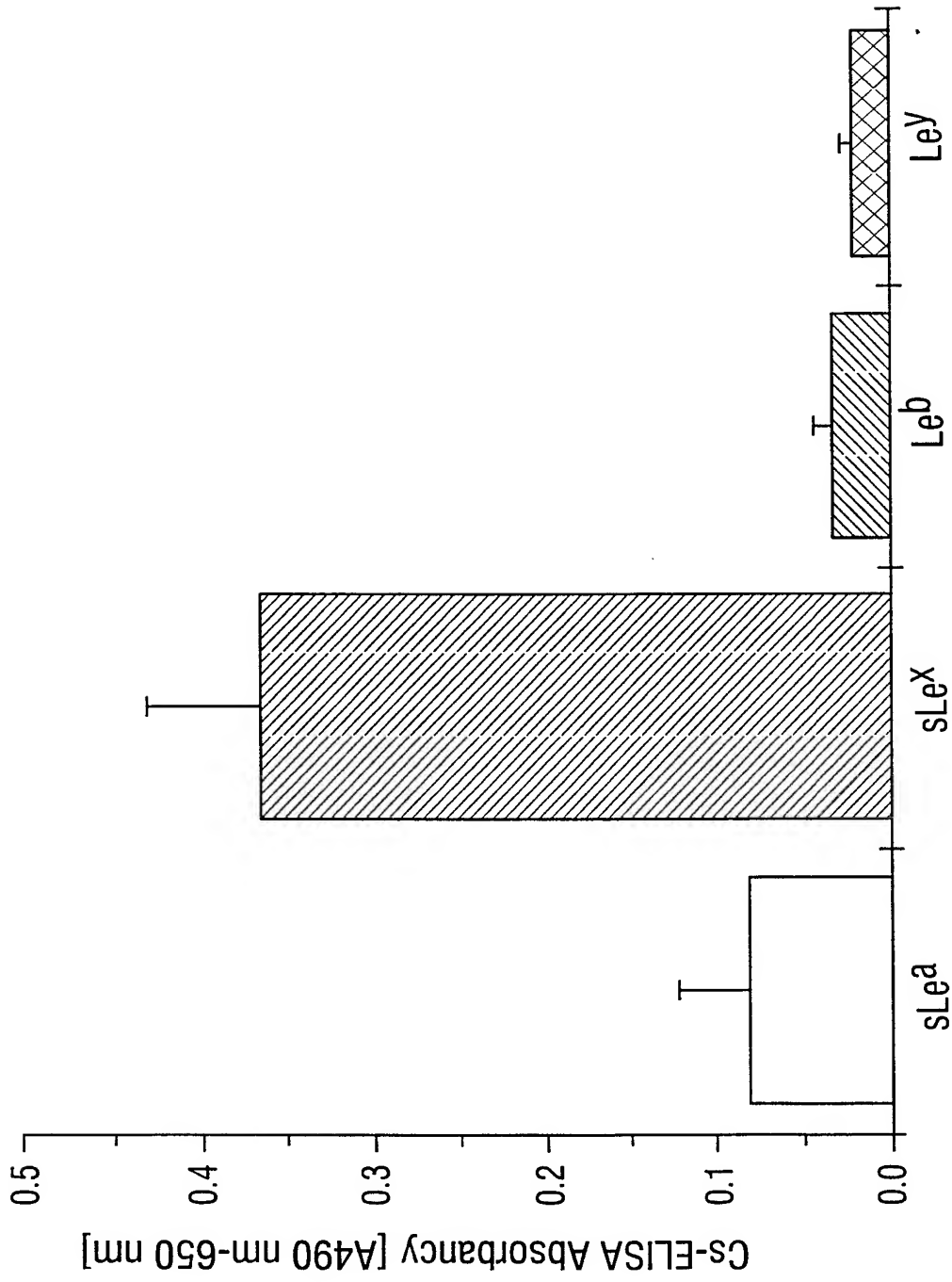
Cs-ELISA Absorbancy [A490 nm-650 nm]
0.0
0.1
0.2
0.3
0.4
0.5
sLe^a
sLe^X
Le^b
Le^Y

FIG. 1D

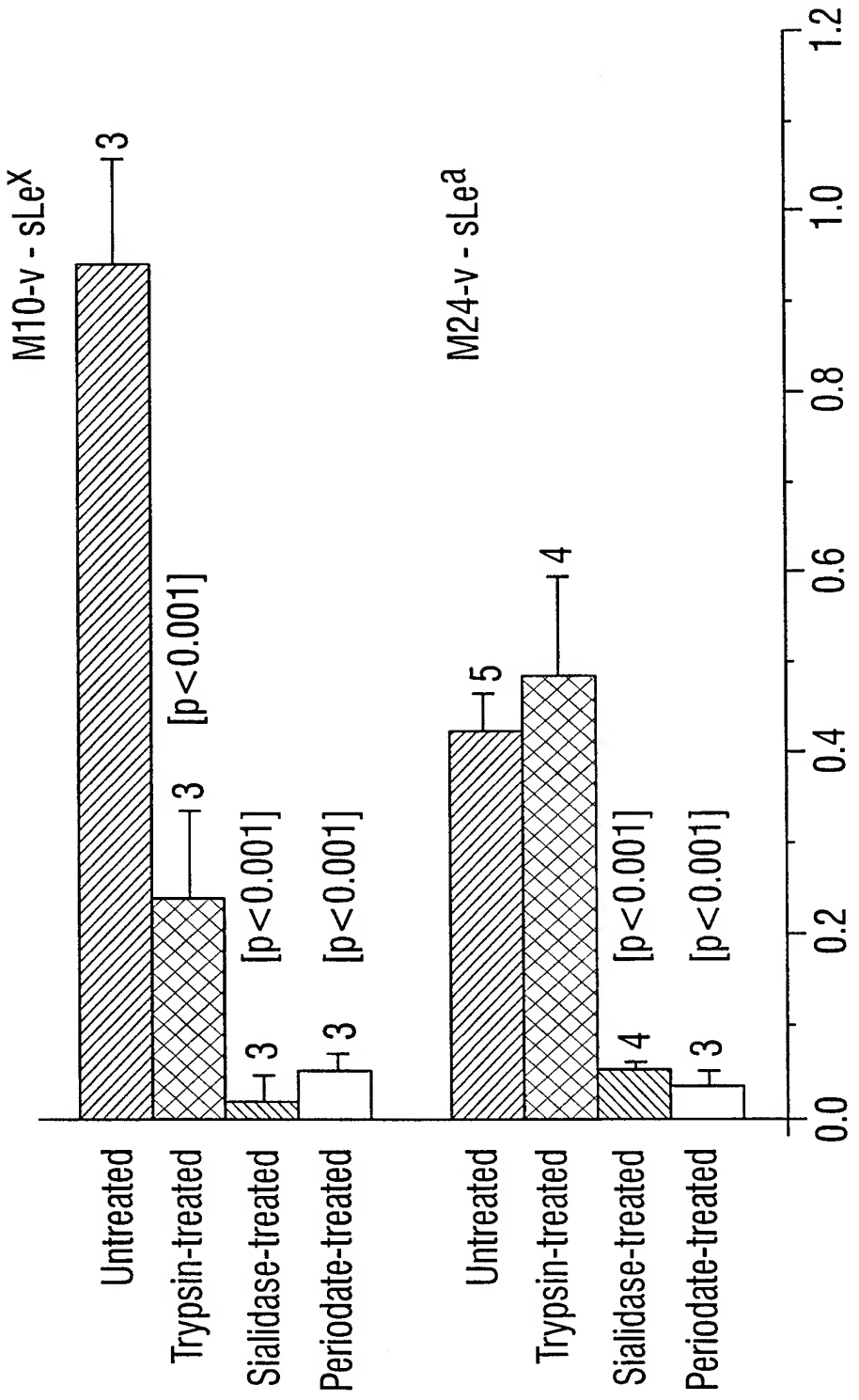
M10-v - sLe^X
M24-v - sLe^a
Untreated
Trypsin-treated
Sialidase-treated
Periodate-treated
Untreated
Trypsin-treated
Sialidase-treated
Periodate-treated
3
3
3
3
5
4
4
3
[p<0.001]
[p<0.001]
[p<0.001]
[p<0.001]
[p<0.001]
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Cs-ELISA [A490 nm-A650 nm]

FIG. 1E

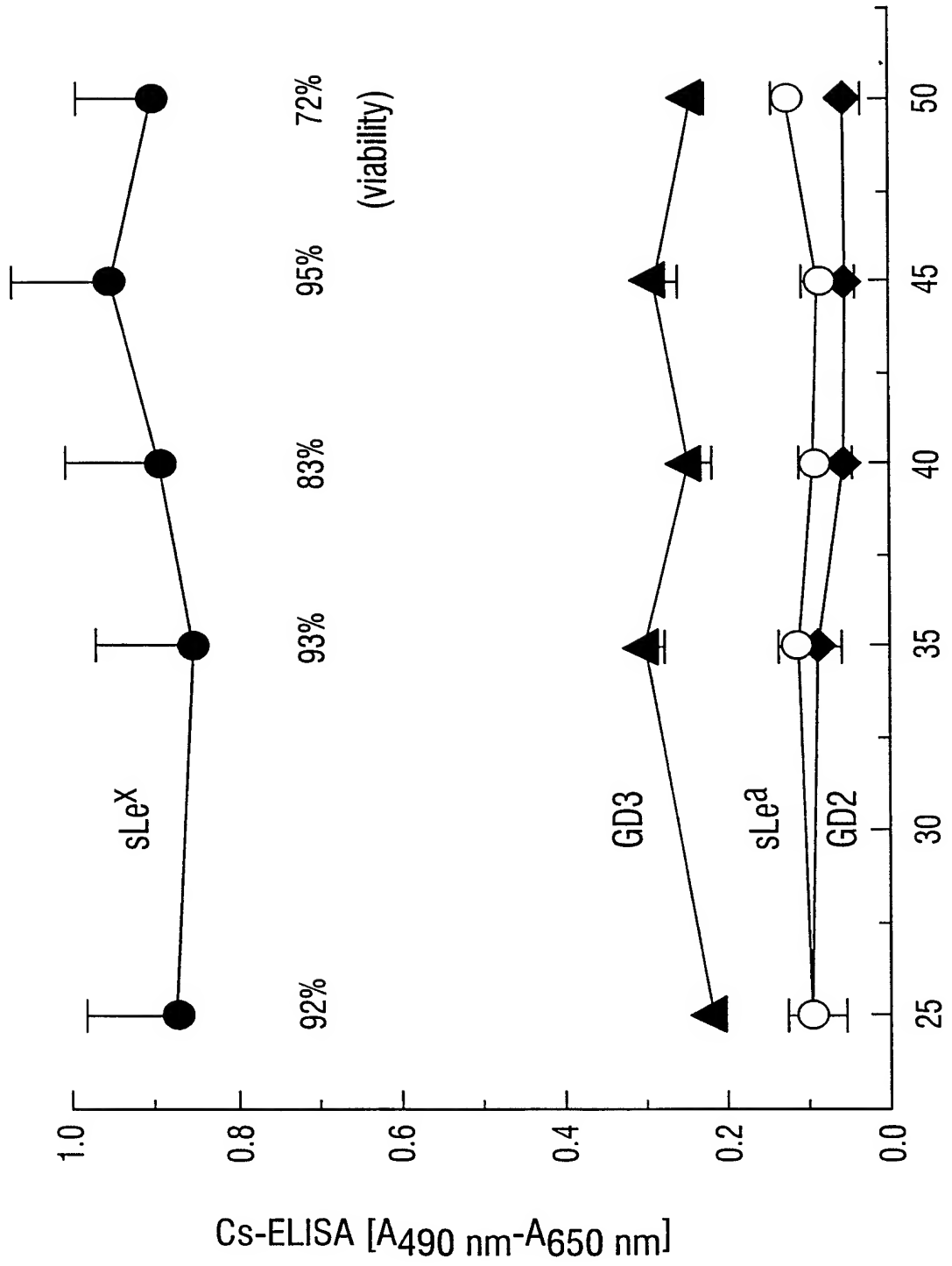
sLe^X
GD3
sLe^a
GD2
92%
93%
83%
95%
72%
(viability)
1.0
0.8
0.6
0.4
0.2
0.0
25
30
35
40
45
50
Cs-ELISA [A490 nm-A650 nm]

FIG. 1F

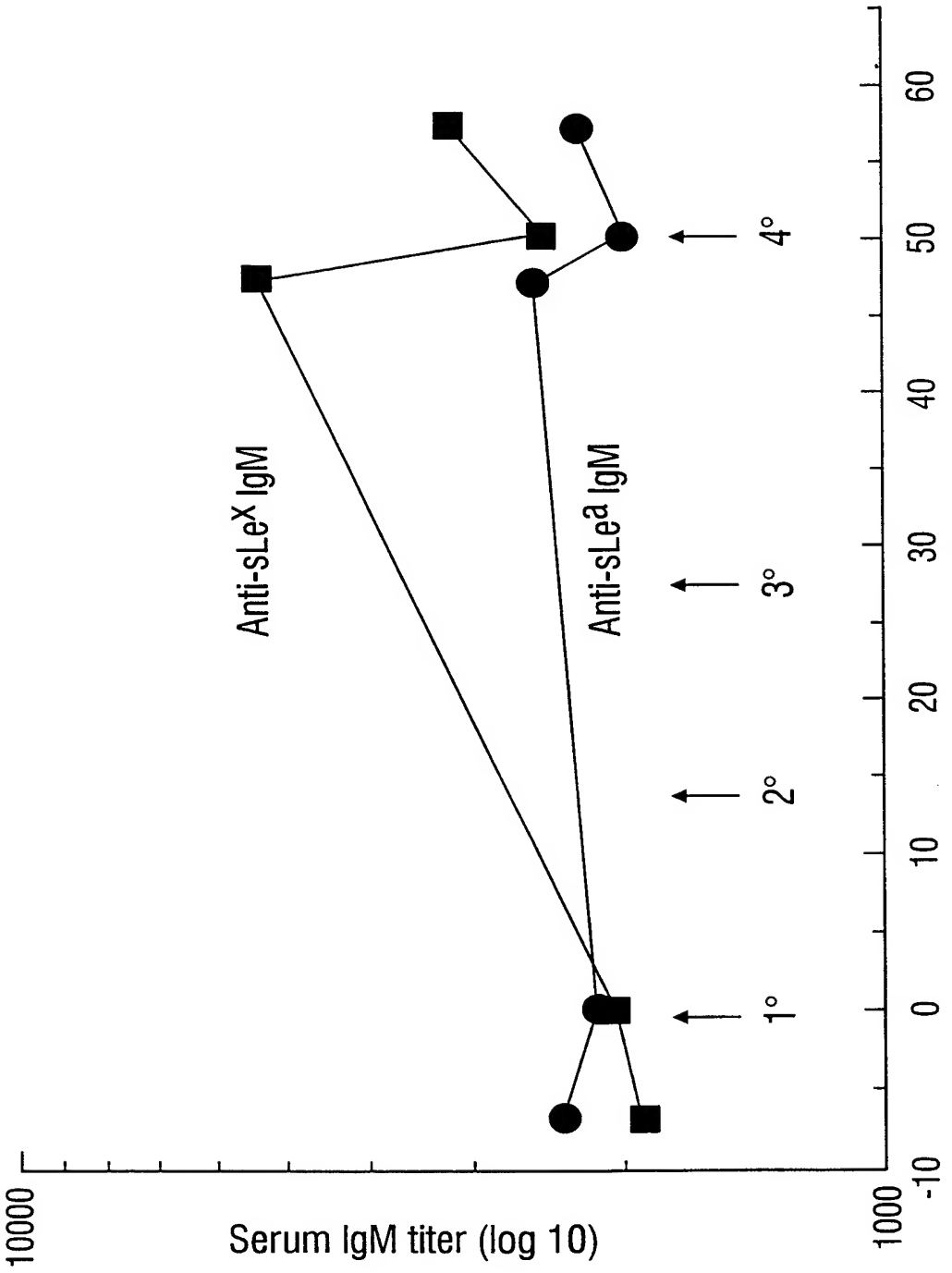
Anti-sLe^X IgM
Anti-sLe^a IgM
1°
2°
3°
4°
10000
1000
Serum IgM titer (log 10)
-10
0
10
20
30
40
50
60

FIG. 2A

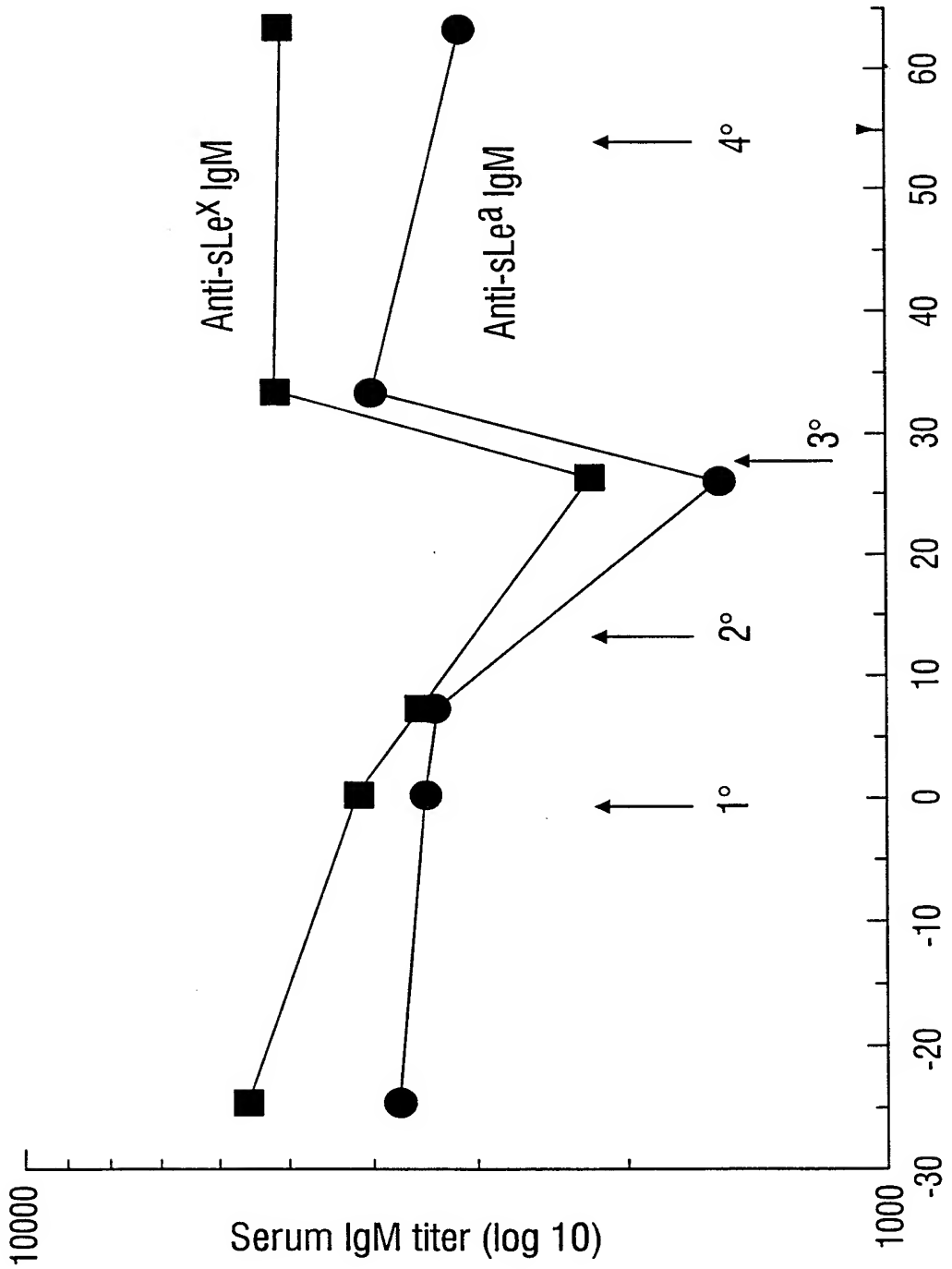
Anti-sLe^X IgM
Anti-sLe^a IgM
4°
3°
2°
1°
10000
1000
Serum IgM titer (log 10)
60
50
40
30
20
10
0
-10
-20
-30

FIG. 2B

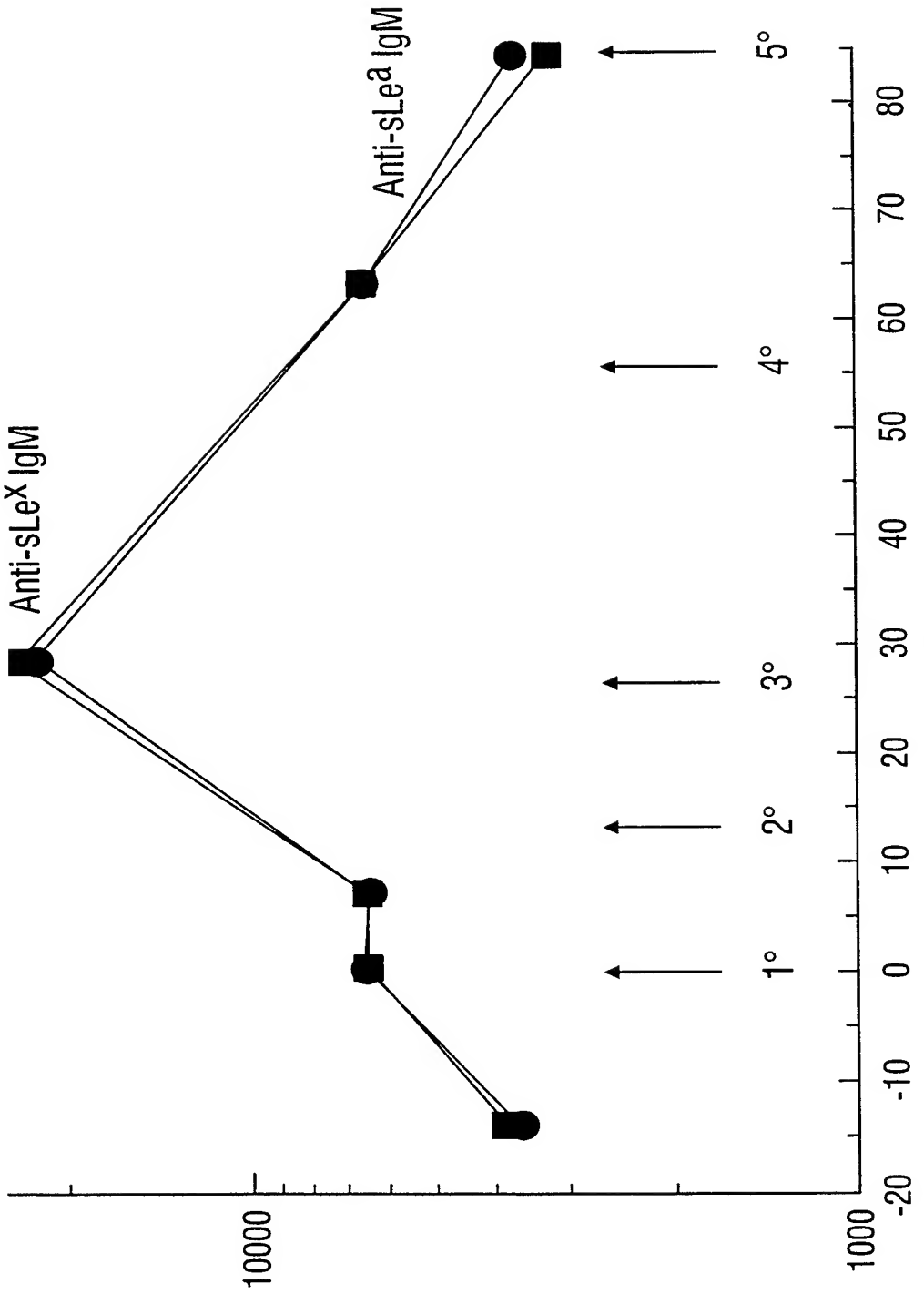
Anti-sLe^X IgM
Anti-sLe^a IgM
1°
2°
3°
4°
5°
10000
1000
-20
-10
0
10
20
30
40
50
60
70
80
Serum IgM titer (log 10)

FIG. 2C

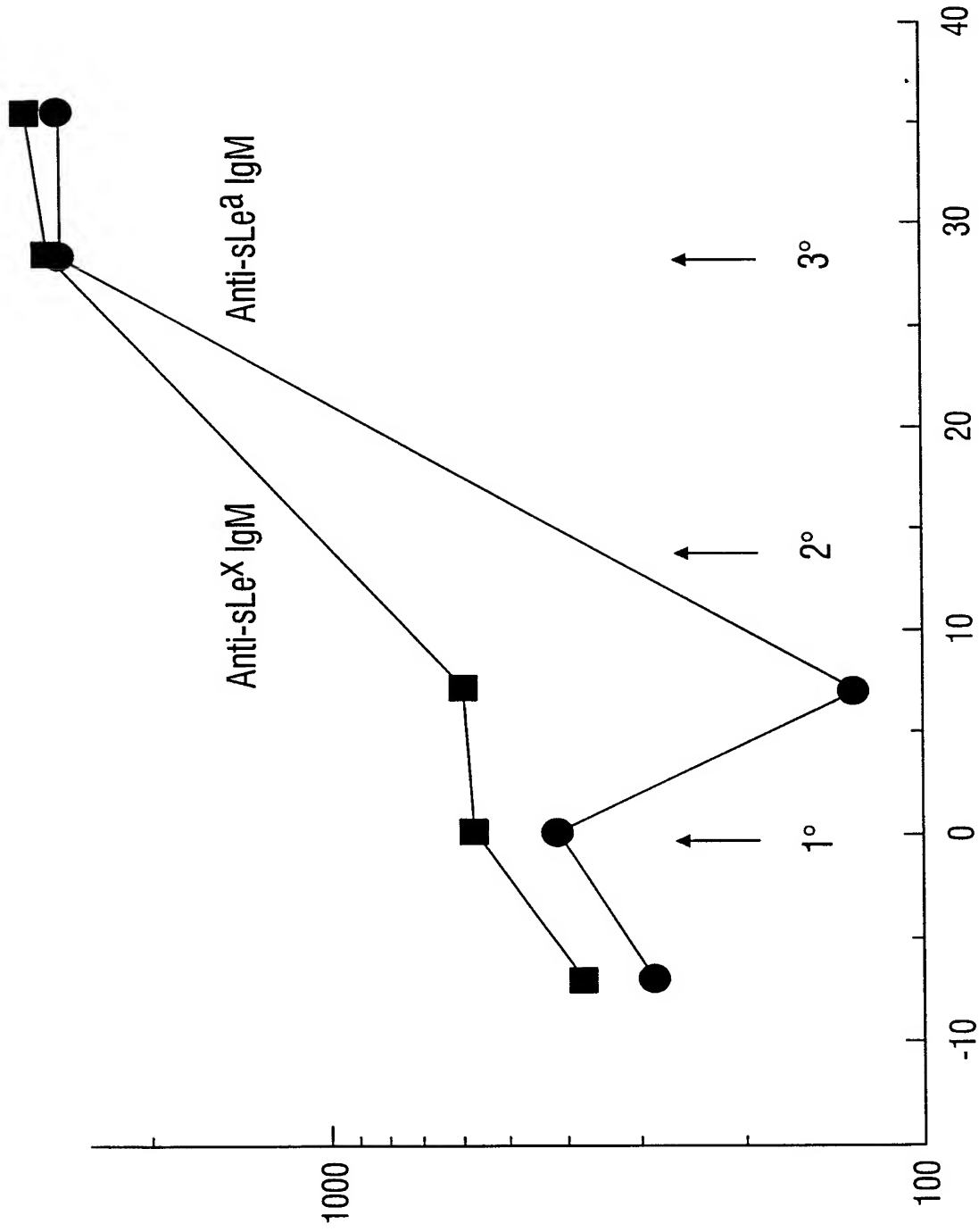
Anti-sLe^a IgM
Anti-sLe^X IgM
1°
2°
3°
40
30
20
10
0
-10
1000
100
Serum IgM titer (log 10)

FIG. 2D

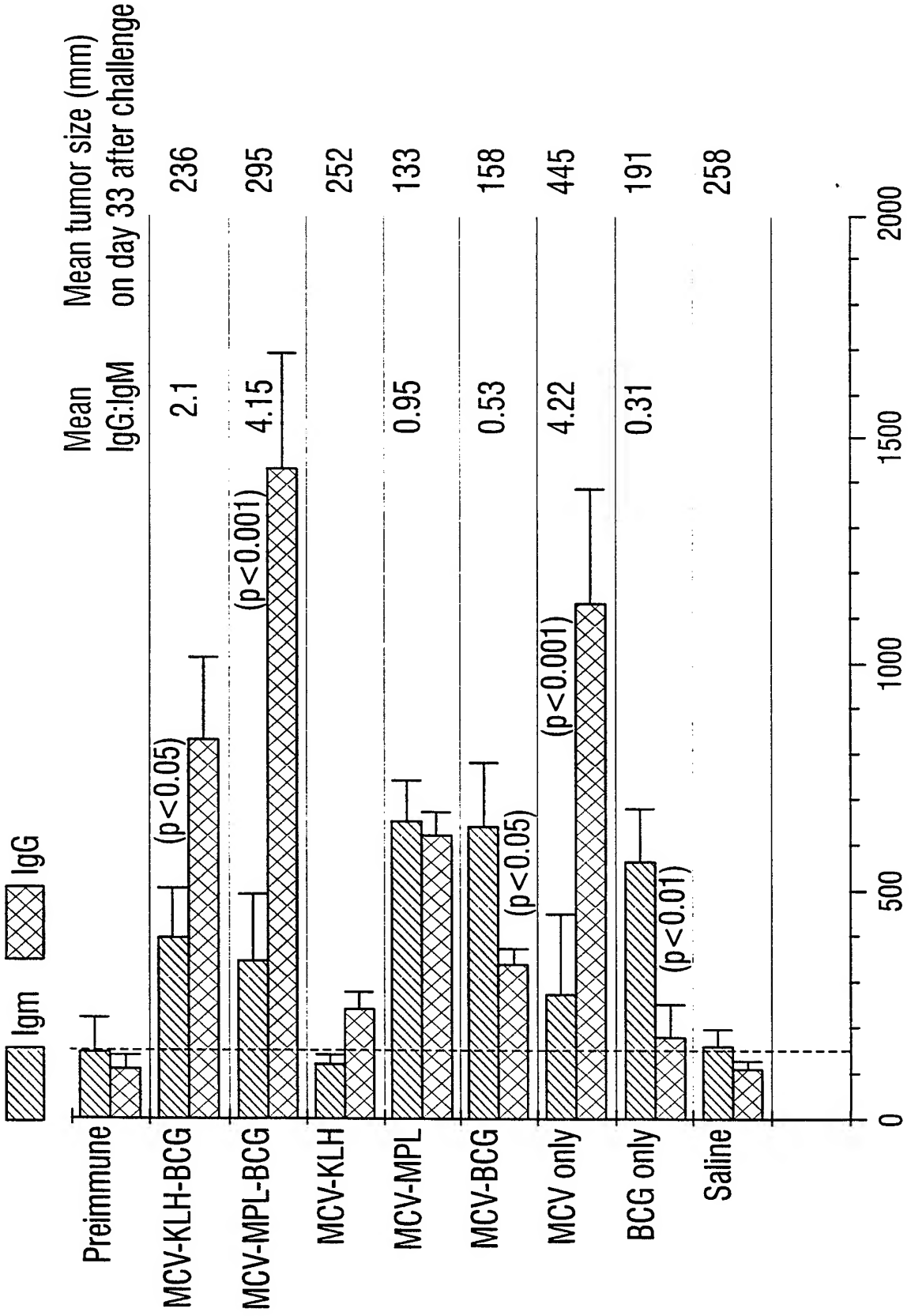
Igm
IgG
Mean IgG:IgM
Mean tumor size (mm) on day 33 after challenge
Preimmune
MCV-KLH-BCG
MCV-MPL-BCG
MCV-KLH
MCV-MPL
MCV-BCG
MCV only
BCG only
Saline
2.1
4.15
0.95
0.53
4.22
0.31
236
295
252
133
158
445
191
258
(p<0.05)
(p<0.001)
(p<0.001)
(p<0.05)
(p<0.01)
0
500
1000
1500
2000

FIG. 3A

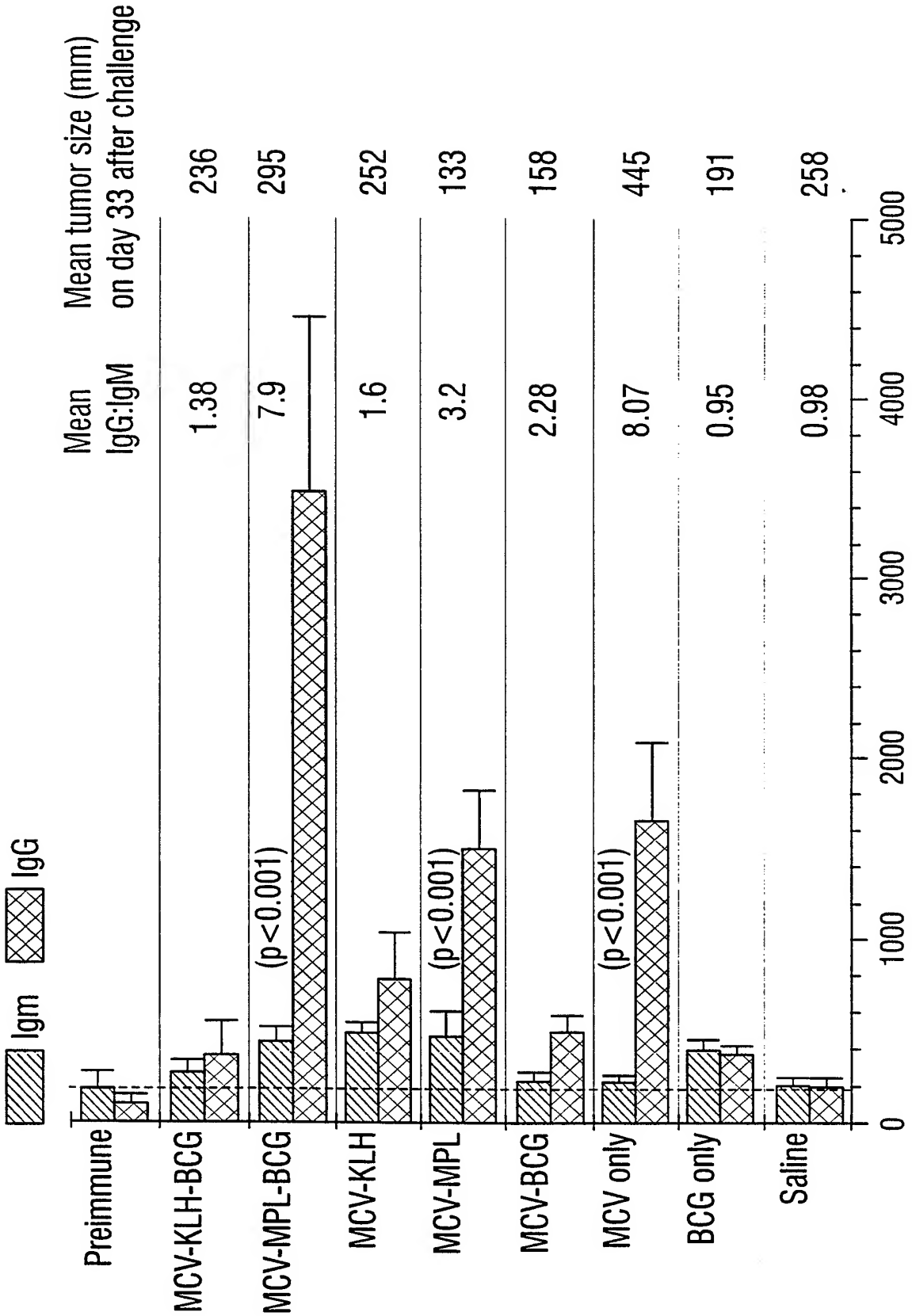
Igm
IgG
Mean IgG:IgM
Mean tumor size (mm) on day 33 after challenge
Preimmune
MCV-KLH-BCG
1.38
236
MCV-MPL-BCG
(p<0.001)
7.9
295
MCV-KLH
1.6
252
MCV-MPL
(p<0.001)
3.2
133
MCV-BCG
2.28
158
MCV only
(p<0.001)
8.07
445
BCG only
0.95
191
Saline
0.98
258
0
1000
2000
3000
4000
5000

FIG. 3B

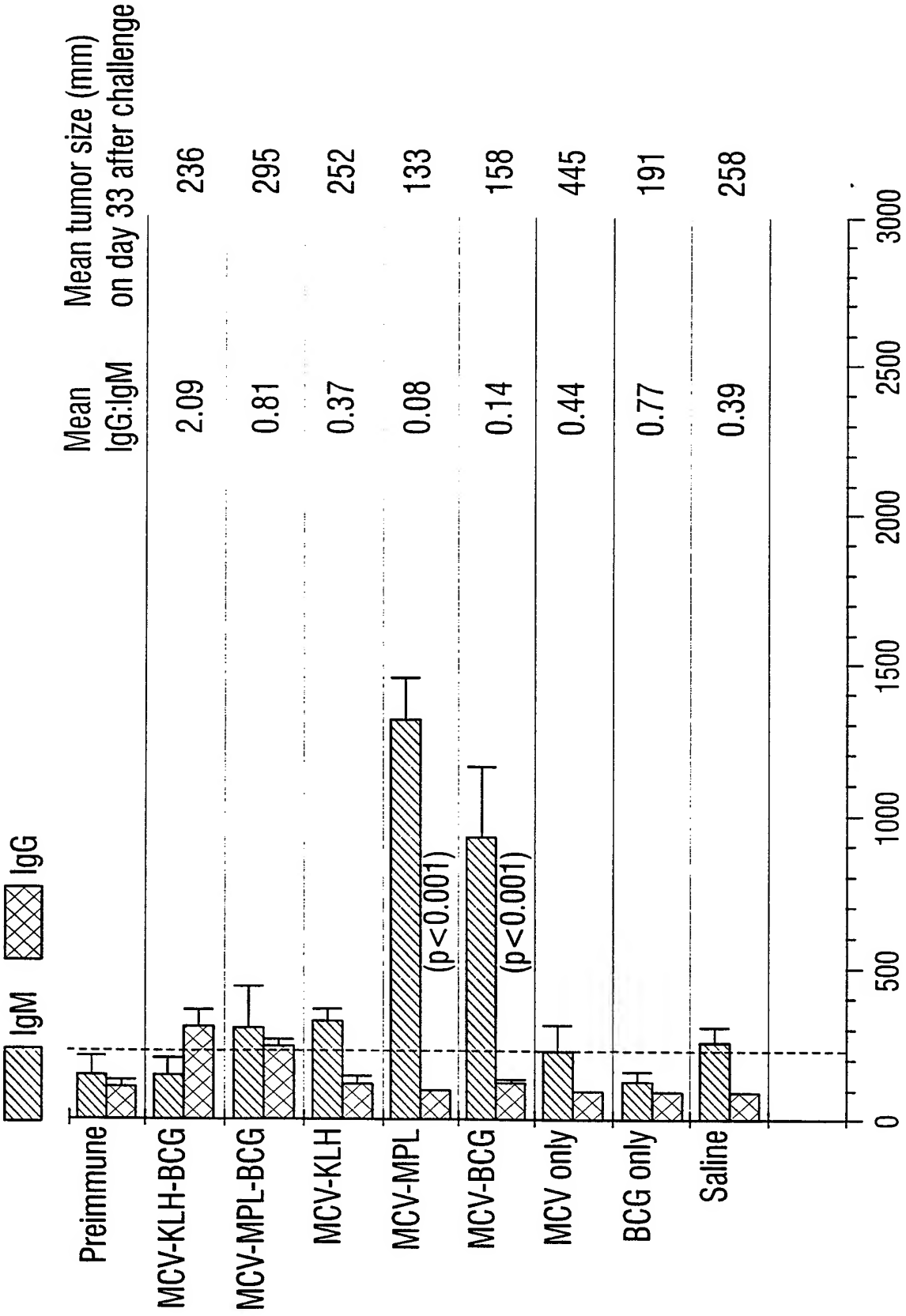
IgM
IgG
Mean IgG:IgM
Mean tumor size (mm) on day 33 after challenge
Preimmune
MCV-KLH-BCG
2.09
236
MCV-MPL-BCG
0.81
295
MCV-KLH
0.37
252
MCV-MPL
0.08
133
(p<0.001)
MCV-BCG
0.14
158
(p<0.001)
MCV only
0.44
445
BCG only
0.77
191
Saline
0.39
258
0
500
1000
1500
2000
2500
3000

FIG. 3C

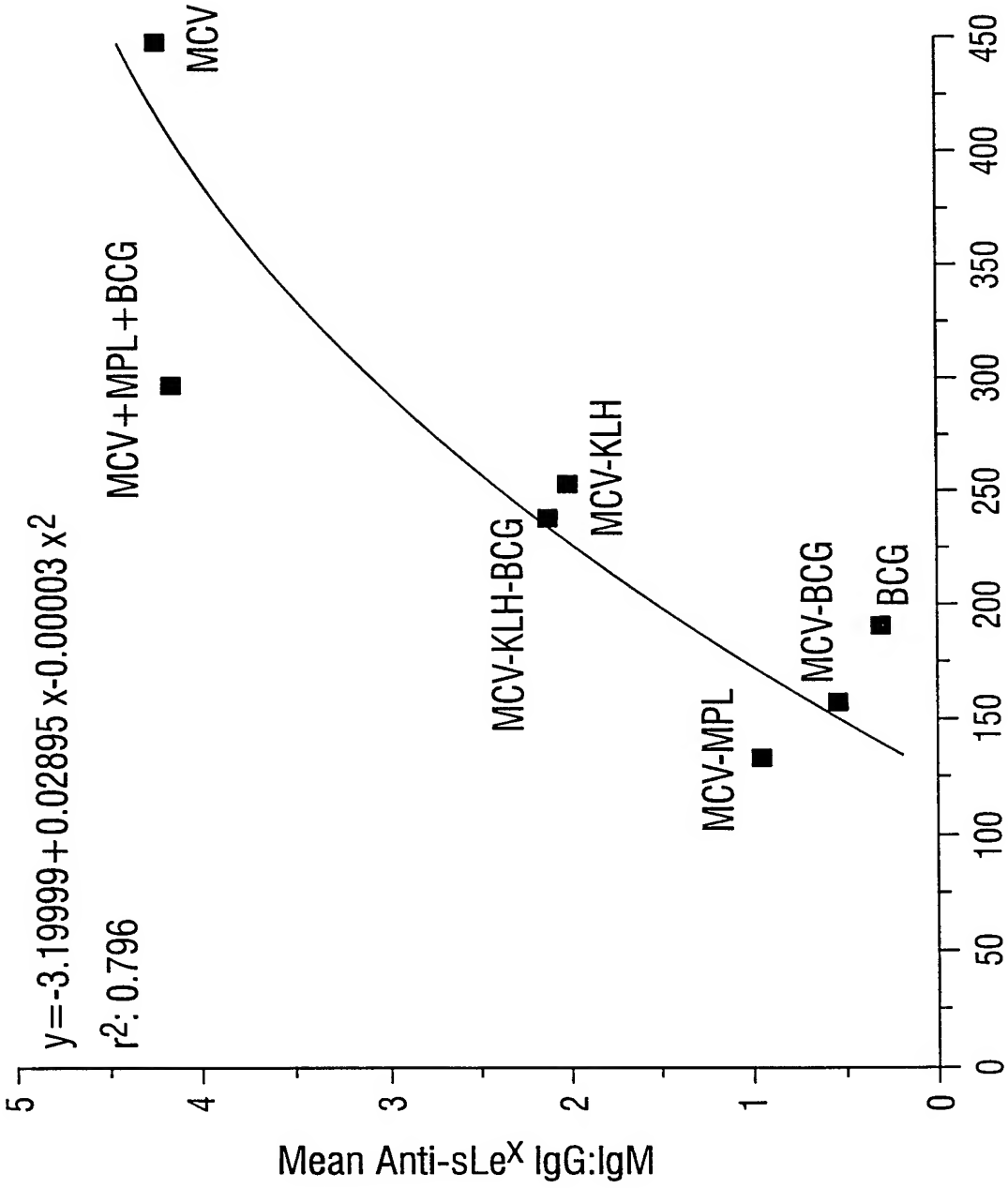
y=-3.19999+0.02895 x-0.00003 x2
r2: 0.796
MCV
MCV+MPL+BCG
MCV-KLH-BCG
MCV-KLH
MCV-MPL
MCV-BCG
BCG
Mean Anti-sLeX IgG:IgM
0
1
2
3
4
5
0
50
100
150
200
250
300
350
400
450

FIG. 4A

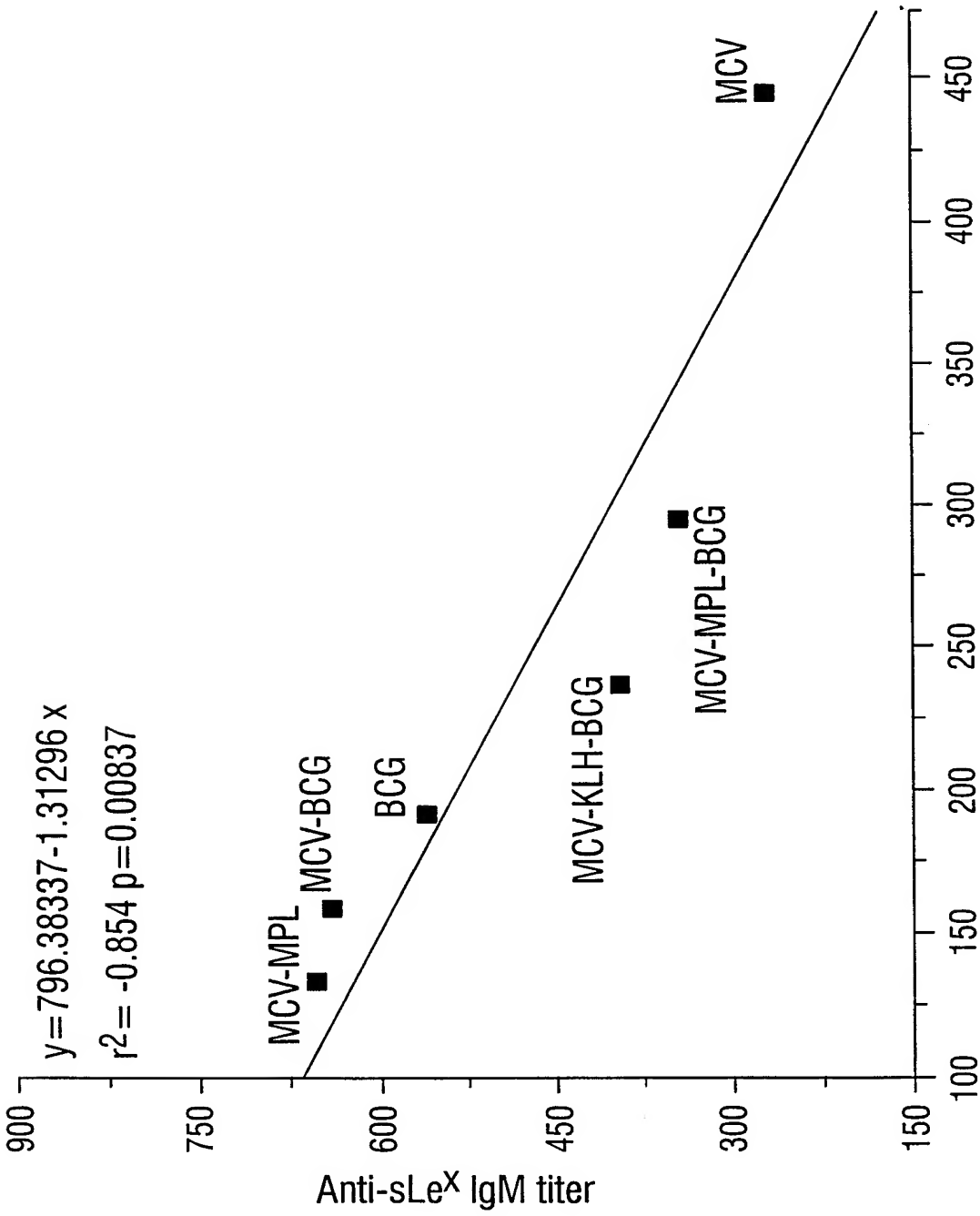
y=796.38337-1.31296 x
r2= -0.854 p=0.00837
MCV-MPL
MCV-BCG
BCG
MCV-KLH-BCG
MCV-MPL-BCG
MCV
900
750
600
450
300
150
100
150
200
250
300
350
400
450
Anti-sLeX IgM titer

FIG. 4B

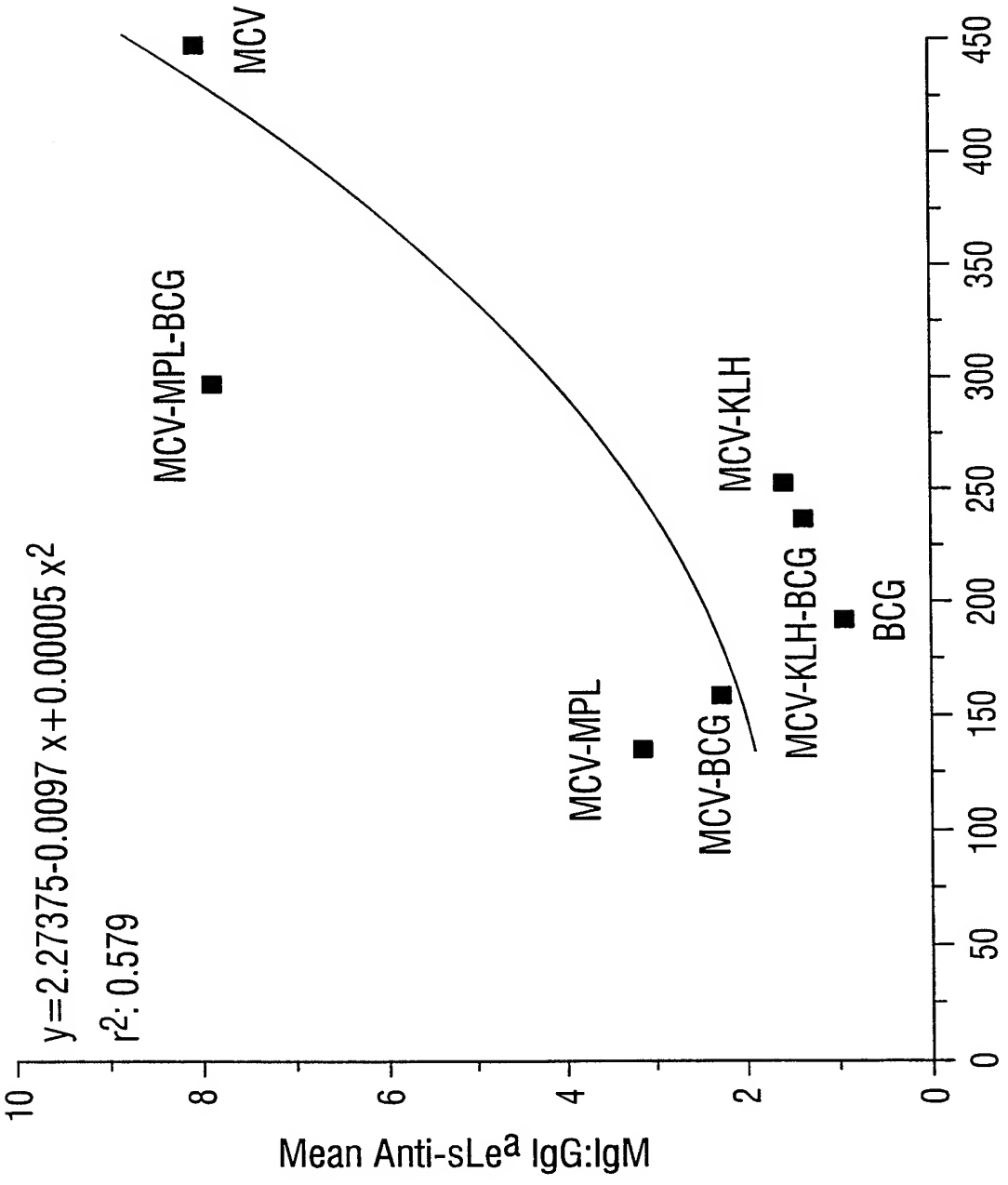
y=2.27375-0.0097 x+0.00005 x2
r2: 0.579
MCV
MCV-MPL-BCG
MCV-KLH
MCV-KLH-BCG
BCG
MCV-MPL
MCV-BCG
Mean Anti-sLea IgG:IgM
0
2
4
6
8
10
0
50
100
150
200
250
300
350
400
450

FIG. 4C

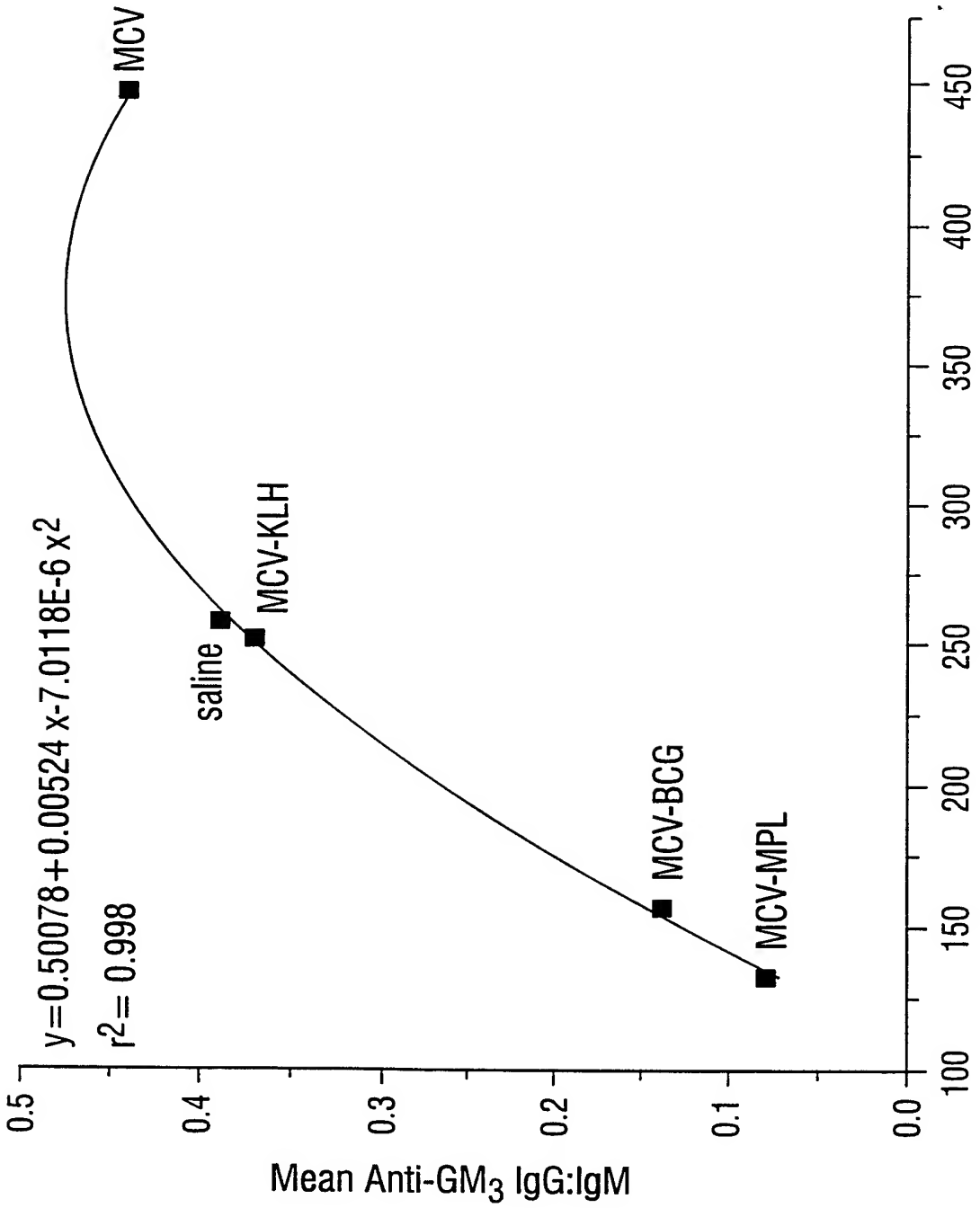
MCV
MCV-KLH
saline
MCV-BCG
MCV-MPL
y=0.50078+0.00524 x-7.0118E-6 x2
r2= 0.998
Mean Anti-GM3 IgG:IgM
0.0
0.1
0.2
0.3
0.4
0.5
100
150
200
250
300
350
400
450

FIG. 4D

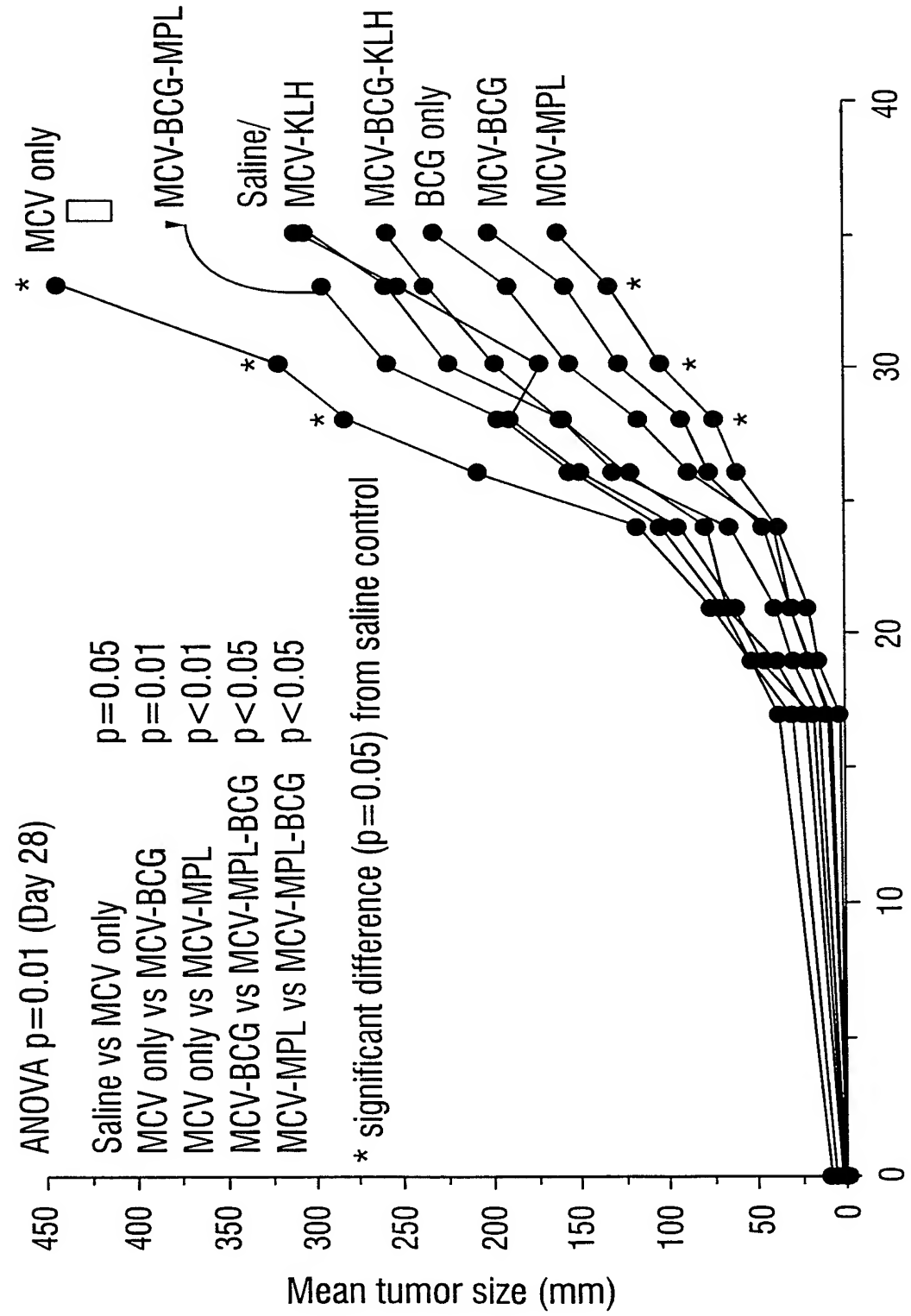
ANOVA p=0.01 (Day 28)
Saline vs MCV only p=0.05
MCV only vs MCV-BCG p=0.01
MCV only vs MCV-MPL p<0.01
MCV-BCG vs MCV-MPL-BCG p<0.05
MCV-MPL vs MCV-MPL-BCG p<0.05
* significant difference (p=0.05) from saline control
* MCV only
MCV-BCG-MPL
Saline/
MCV-KLH
MCV-BCG-KLH
BCG only
MCV-BCG
MCV-MPL
Mean tumor size (mm)
450
400
350
300
250
200
150
100
50
0
0
10
20
30
40

FIG. 5A

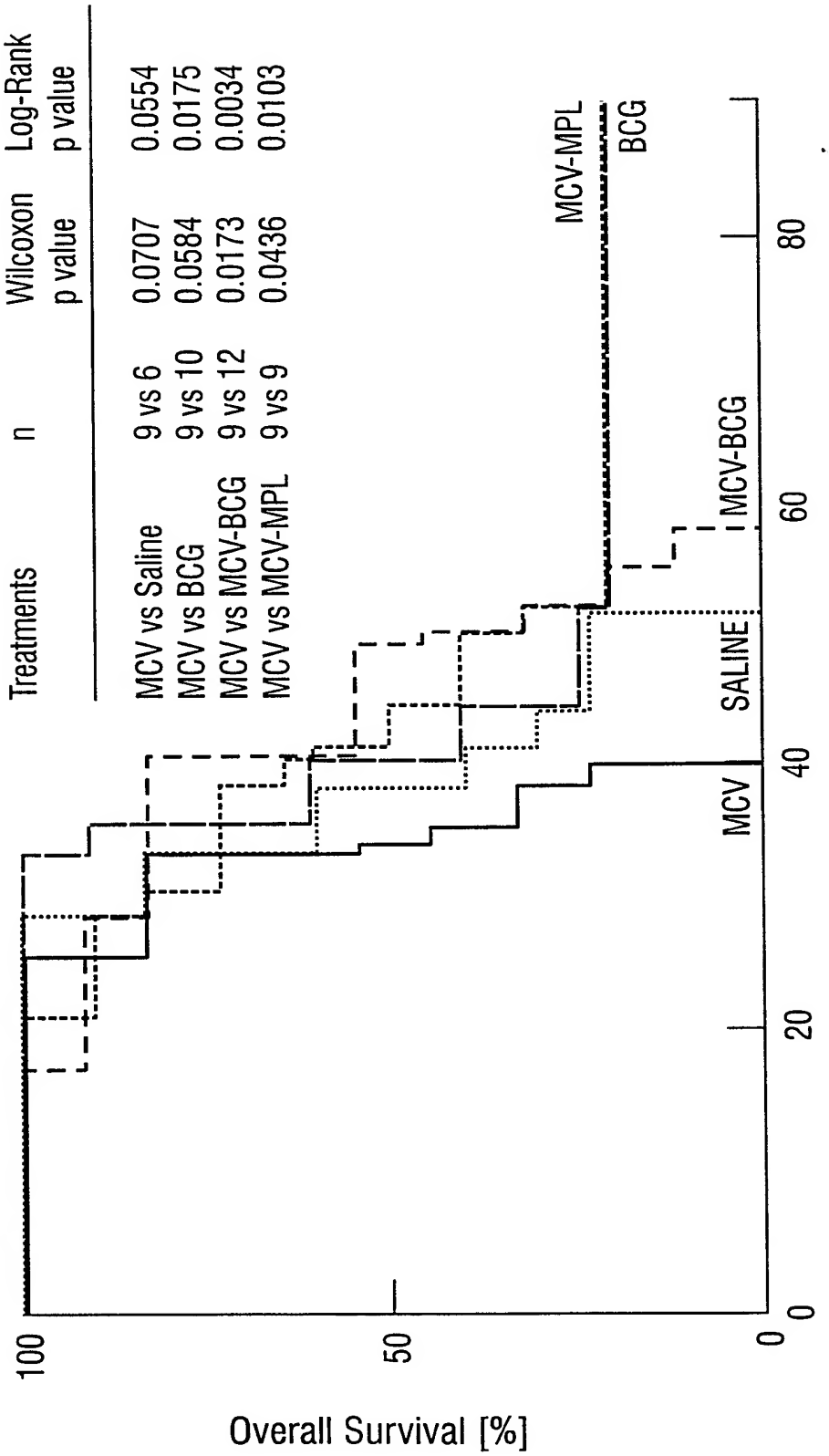
Treatments
n
Wilcoxon p value
Log-Rank p value
MCV vs Saline 9 vs 6 0.0707 0.0554
MCV vs BCG 9 vs 10 0.0584 0.0175
MCV vs MCV-BCG 9 vs 12 0.0173 0.0034
MCV vs MCV-MPL 9 vs 9 0.0436 0.0103
MCV-MPL
BCG
MCV-BCG
SALINE
MCV
0
20
40
60
80
100
50
0
Overall Survival [%]

FIG. 5B

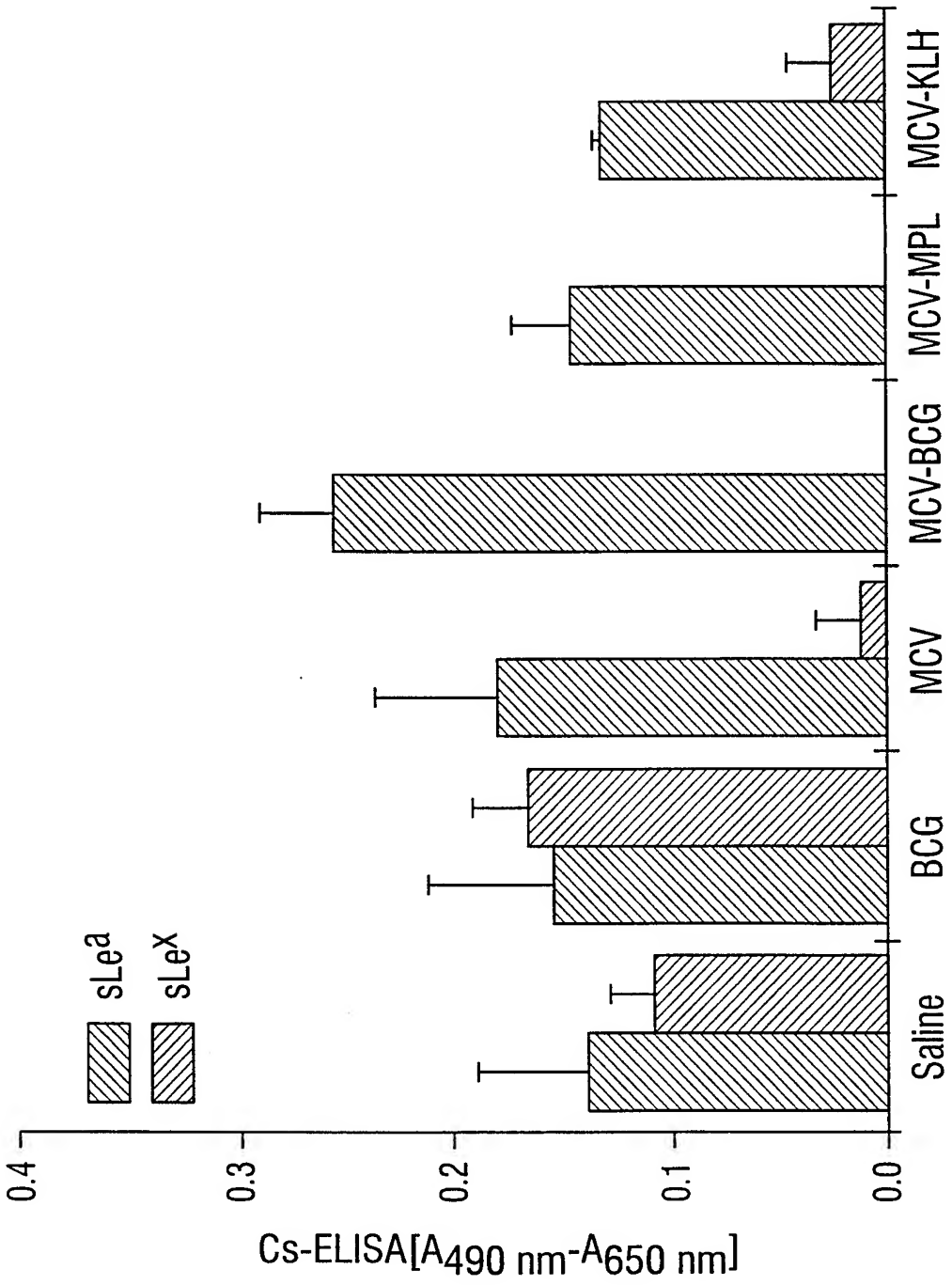
sLe^a
sLe^X
Saline
BCG
MCV
MCV-BCG
MCV-MPL
MCV-KLH
0.0
0.1
0.2
0.3
0.4
Cs-ELISA[A490 nm-A650 nm]

FIG. 6A

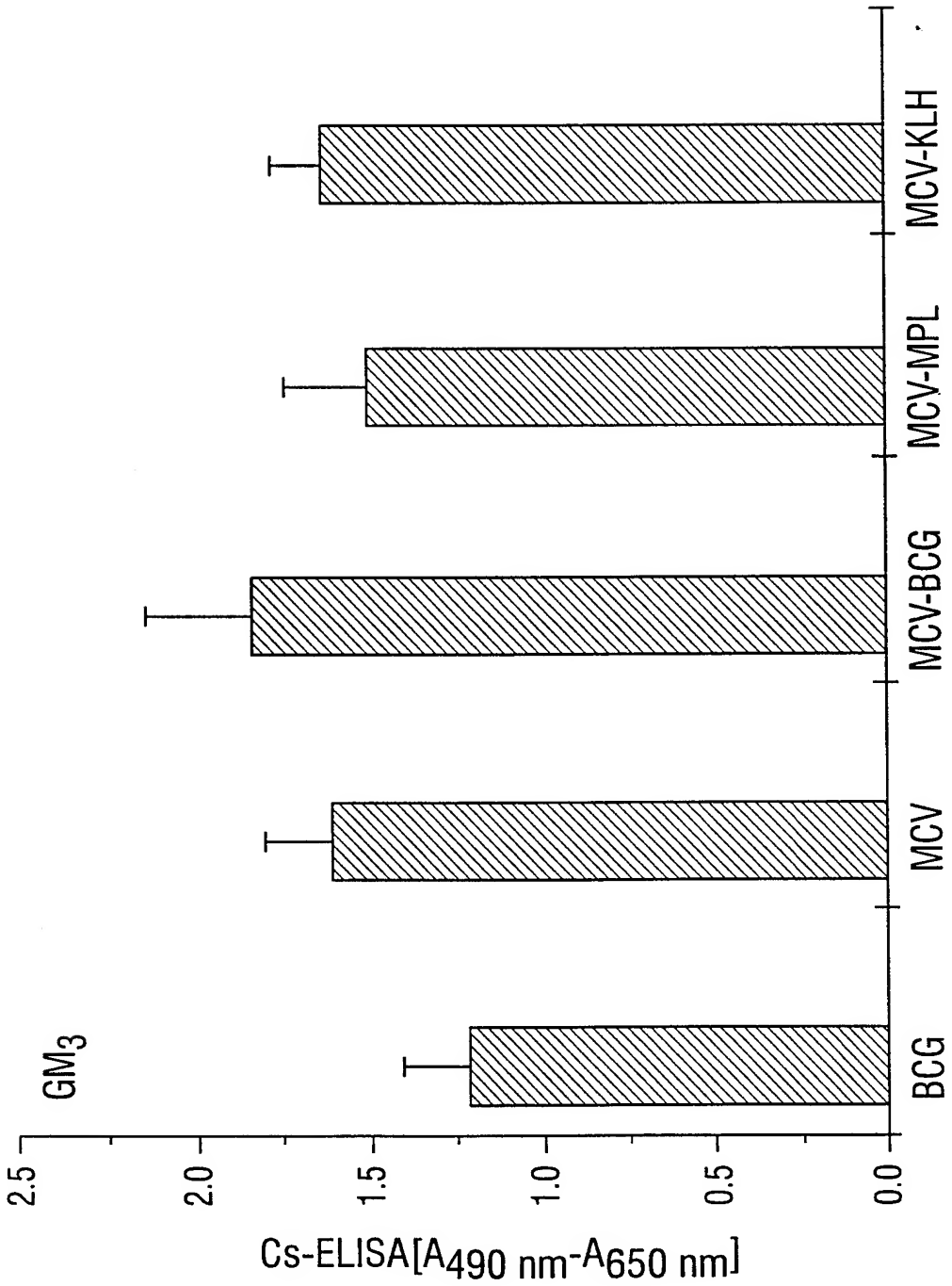
GM3
BCG
MCV
MCV-BCG
MCV-MPL
MCV-KLH
2.5
2.0
1.5
1.0
0.5
0.0
Cs-ELISA[A490 nm-A650 nm]

FIG. 6B

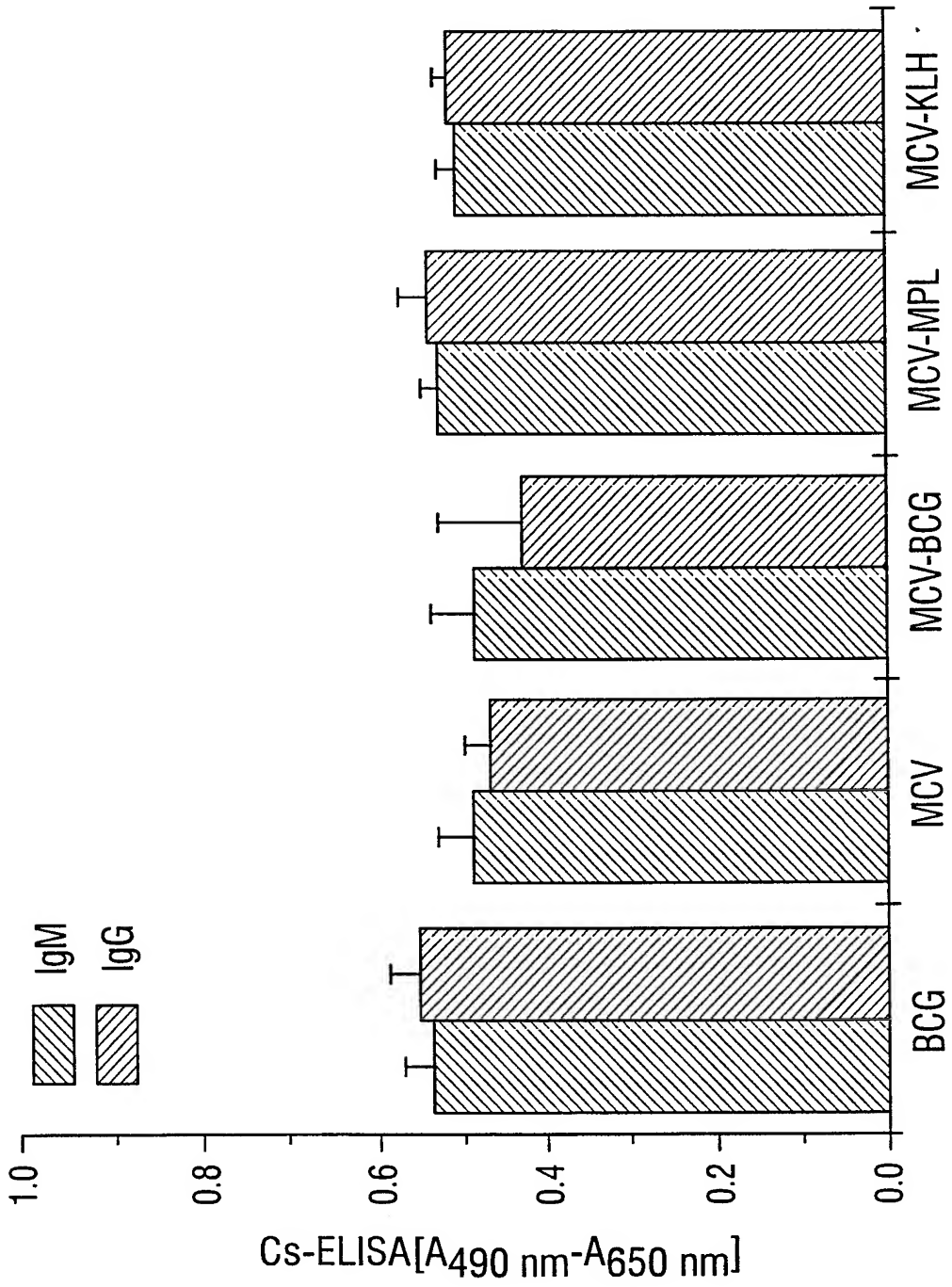
IgM
IgG
BCG
MCV
MCV-BCG
MCV-MPL
MCV-KLH
1.0
0.8
0.6
0.4
0.2
0.0
Cs-ELISA[A490 nm-A650 nm]

FIG. 6C

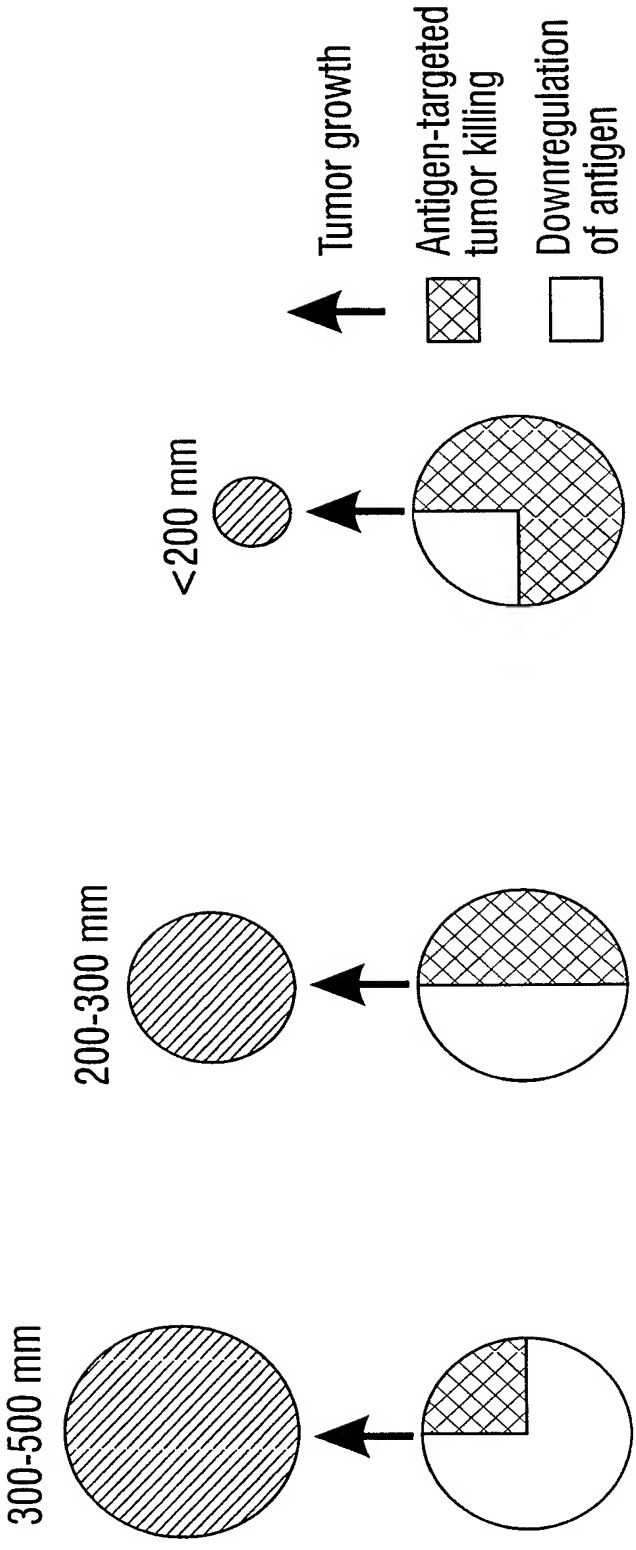
Tumor growth
Antigen-targeted tumor killing
Downregulation of antigen
<200 mm
Mice #3 IgG:IgM (1:5) Million Tumor Cells
200-300 mm
Mice #2 IgG:IgM (1:1) Million Tumor Cells
300-500 mm
Mice #1 IgG:IgM (5:1) Million Tumor Cells

FIG. 7

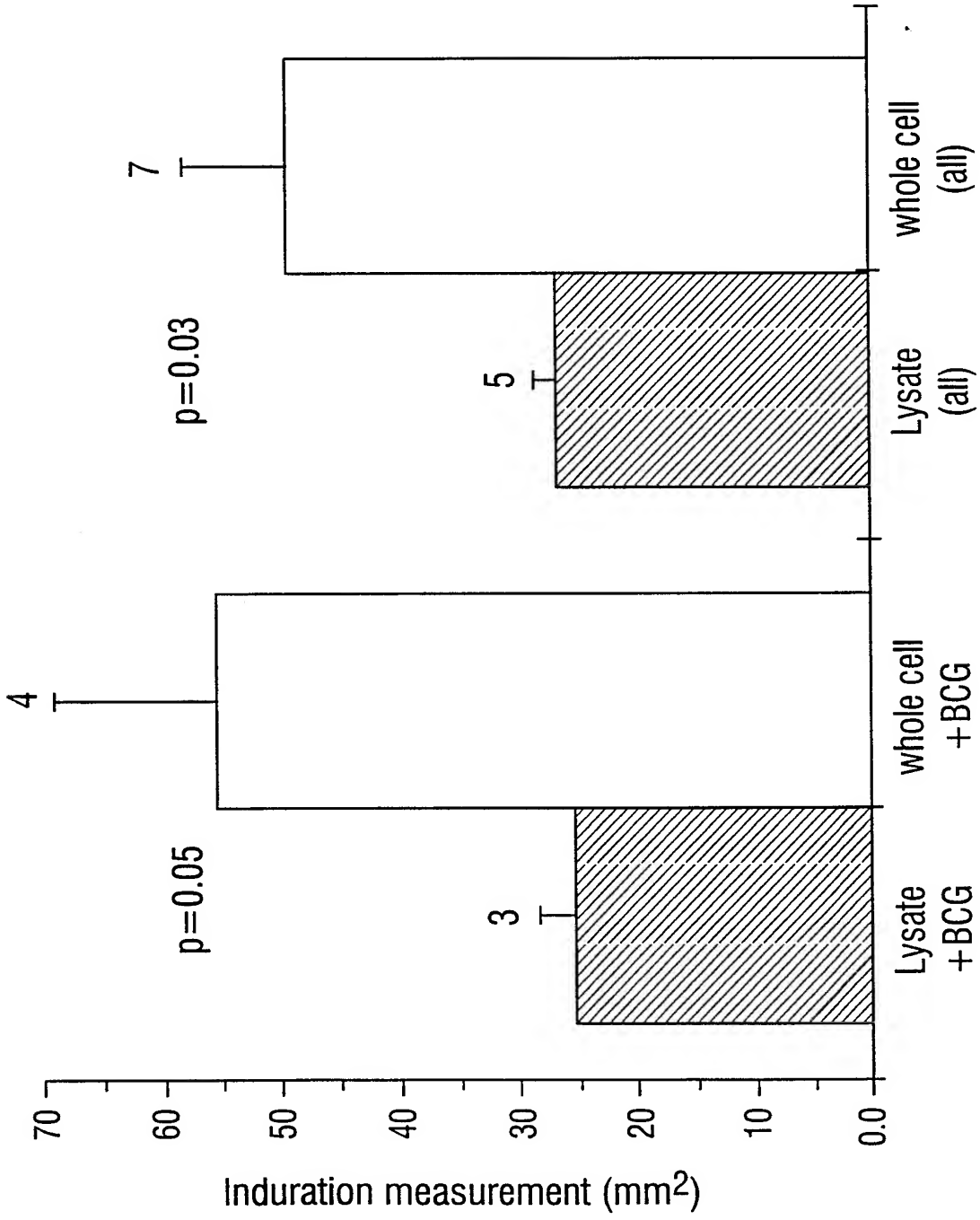
Induration measurement (mm2)
70
60
50
40
30
20
10
0.0
Lysate +BCG
whole cell +BCG
Lysate (all)
whole cell (all)
3
4
5
7
p=0.05
p=0.03

FIG. 8A

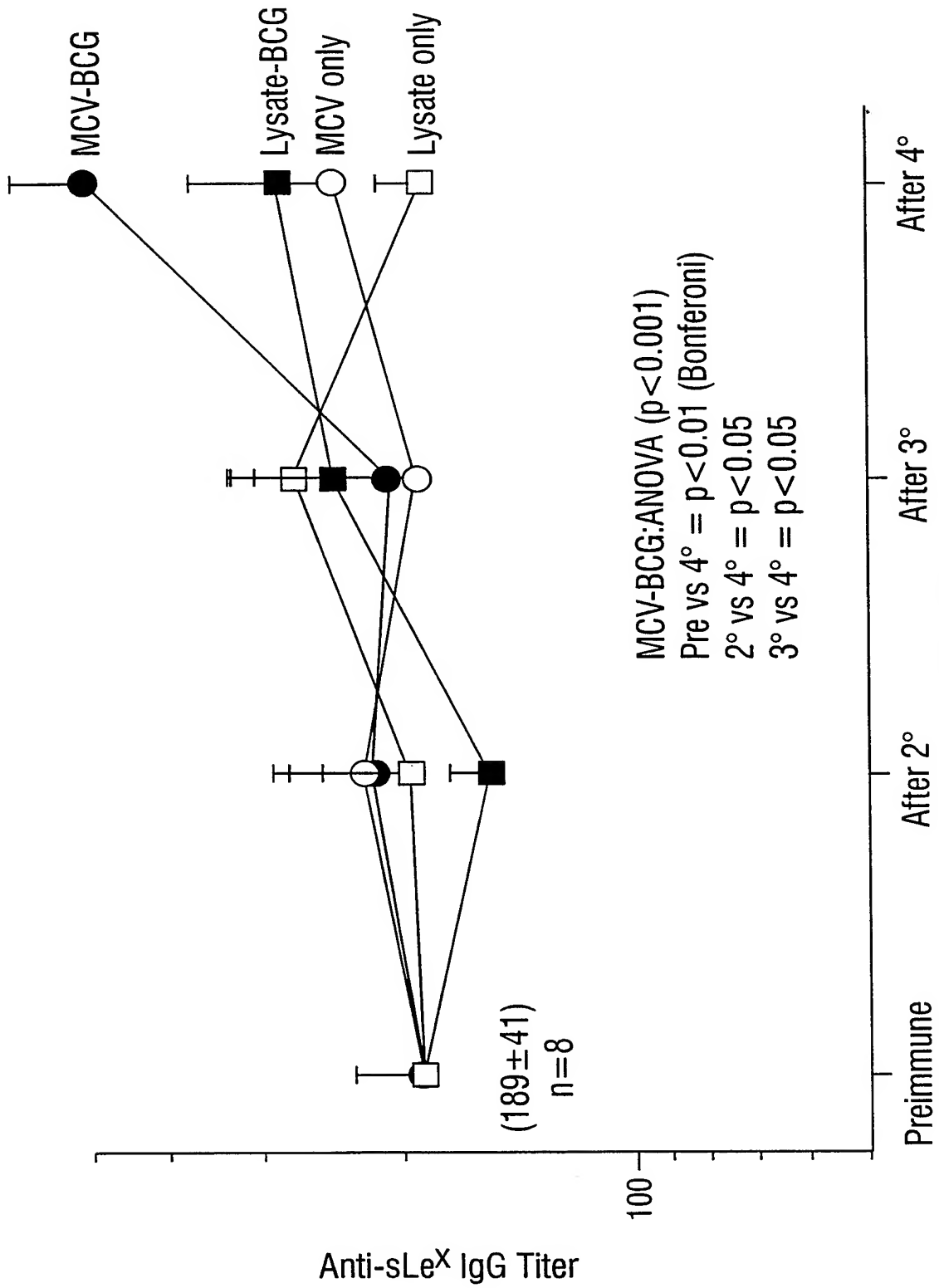
MCV-BCG
Lysate-BCG
MCV only
Lysate only
MCV-BCG:ANOVA (p<0.001)
Pre vs 4° = p<0.01 (Bonferoni)
2° vs 4° = p<0.05
3° vs 4° = p<0.05
(189±41)
n=8
100
Preimmune
After 2°
After 3°
After 4°
Anti-sLe^X IgG Titer

FIG. 8B

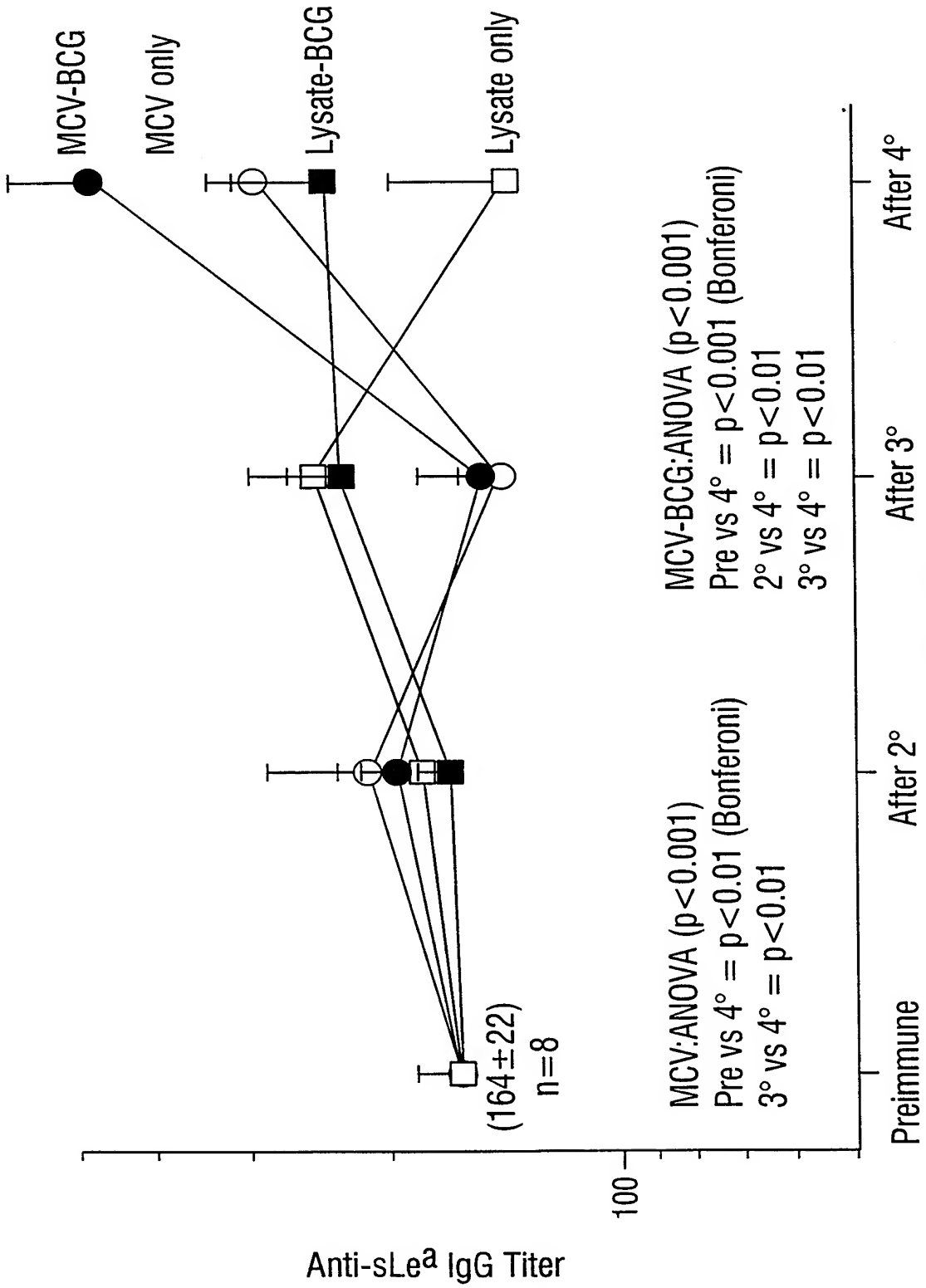
MCV-BCG
MCV only
Lysate-BCG
Lysate only
(164±22)
n=8
MCV:ANOVA (p<0.001)
Pre vs 4° = p<0.01 (Bonferoni)
3° vs 4° = p<0.01
MCV-BCG:ANOVA (p<0.001)
Pre vs 4° = p<0.001 (Bonferoni)
2° vs 4° = p<0.01
3° vs 4° = p<0.01
100
Anti-sLe^a IgG Titer
Preimmune
After 2°
After 3°
After 4°

FIG. 8C

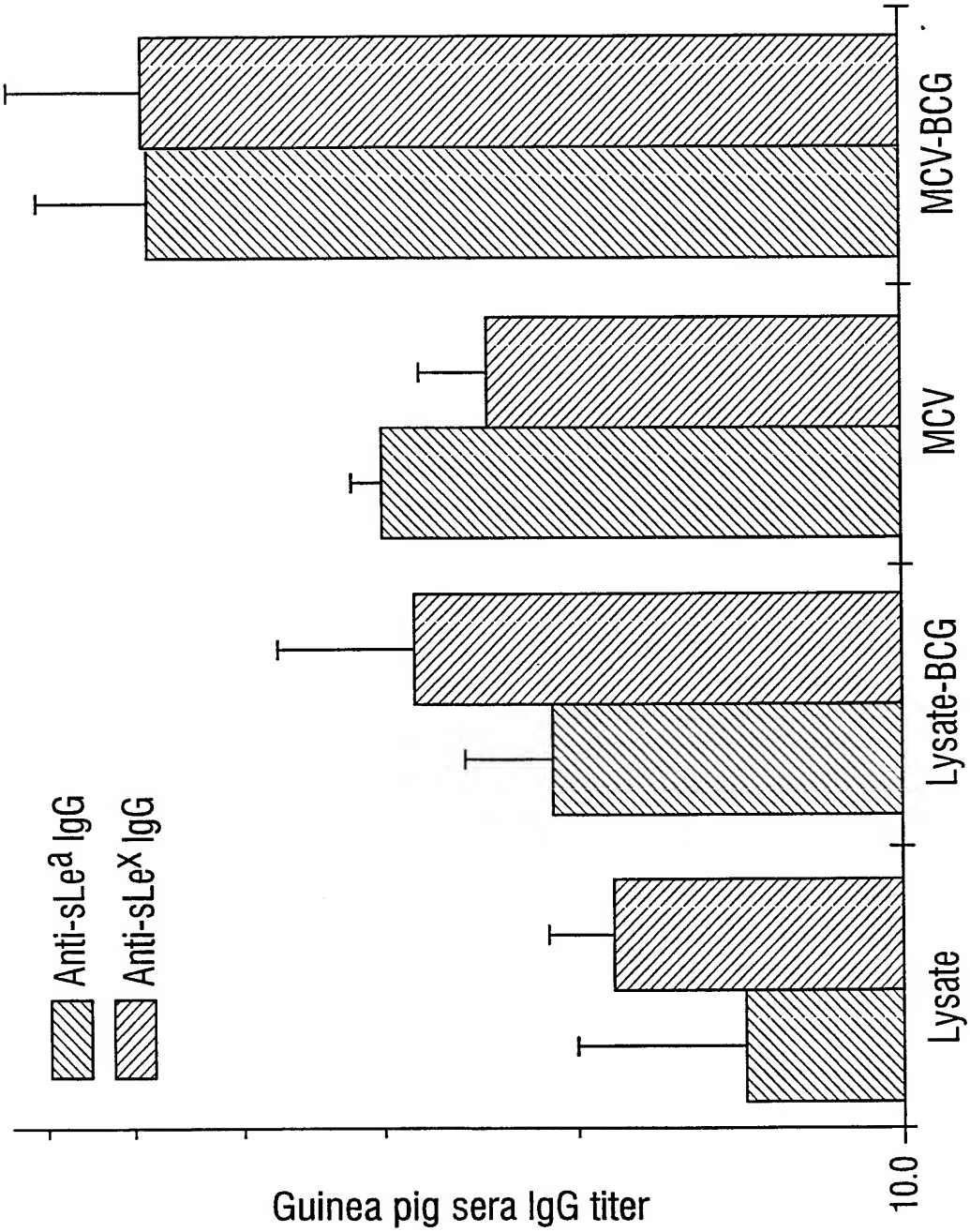
MCV-BCG
MCV
Lysate-BCG
Lysate
Anti-sLe^a IgG
Anti-sLe^X IgG
10.0
Guinea pig sera IgG titer

FIG. 8D

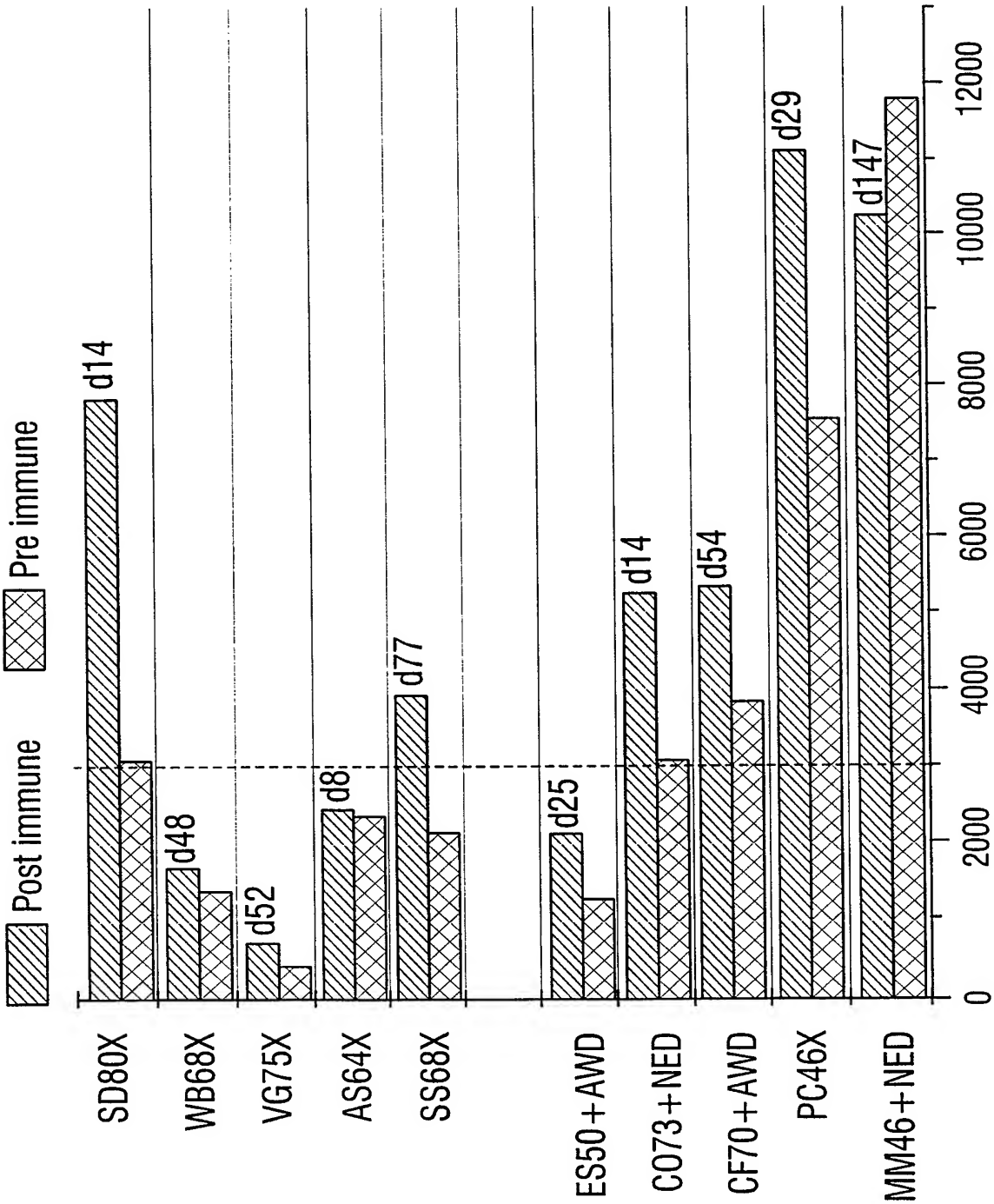
Post immune
Pre immune
SD80X
WB68X
VG75X
AS64X
SS68X
ES50+AWD
CO73+NED
CF70+AWD
PC46X
MM46+NED
d14
d48
d52
d8
d77
d25
d14
d54
d29
d147
0
2000
4000
6000
8000
10000
12000

FIG. 9A

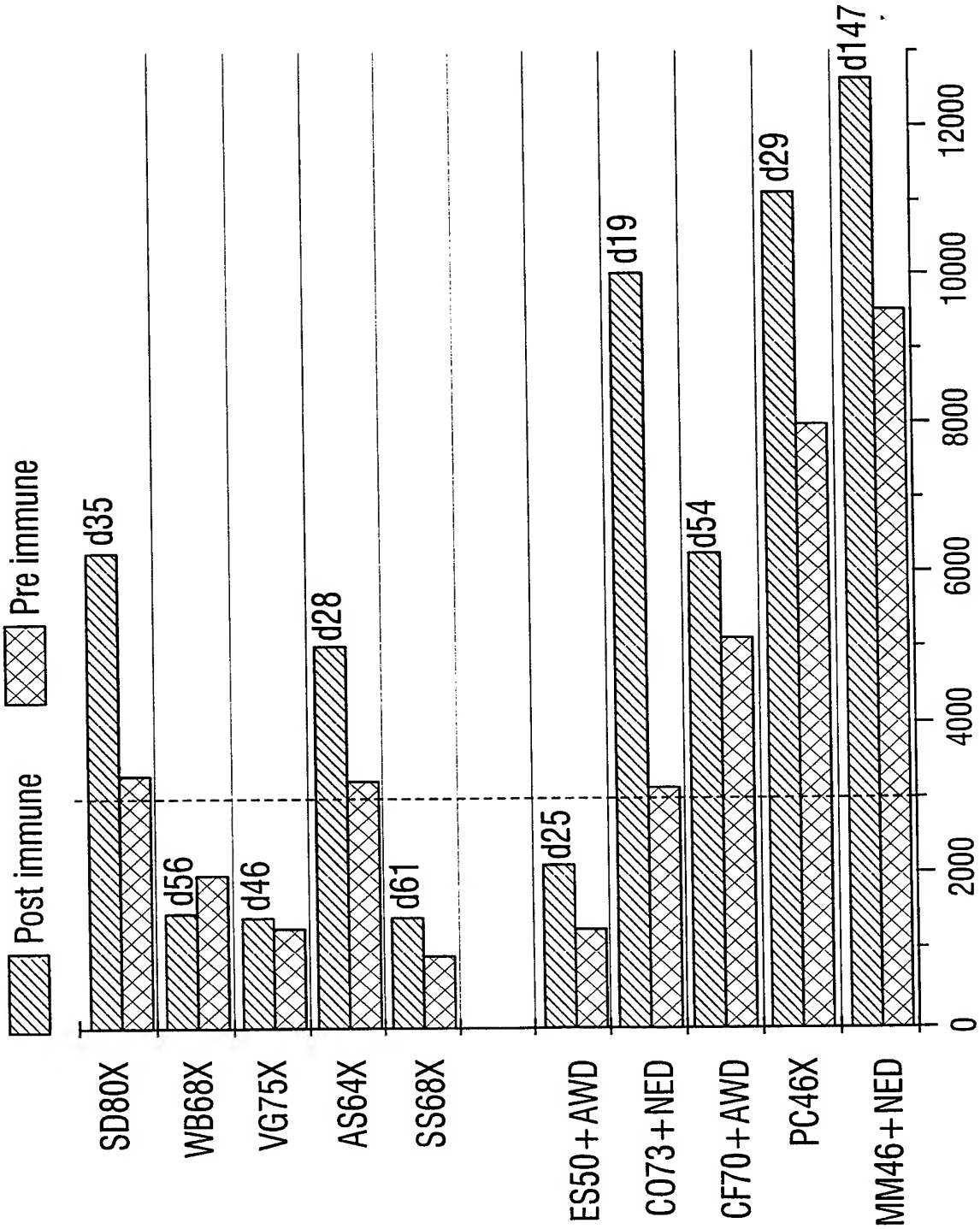
Post immune
Pre immune
SD80X
WB68X
VG75X
AS64X
SS68X
ES50+AWD
CO73+NED
CF70+AWD
PC46X
MM46+NED
d35
d56
d46
d28
d61
d25
d19
d54
d29
d147
0
2000
4000
6000
8000
10000
12000

FIG. 9B